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13. ABSTRACT ***(Maximum 200 Words)***

Changes in arachidonic acid (AA) metabolism may be crucial in development or progression of prostate adenocarcinoma (Pca). By immunohistochemistry, western blots, Northern blots, and enzyme assays, we have detected the uniform expression of a novel lipoxygenase, 15-LOX-2, in benign prostate. 15-LOX-2 and 15-HETE formation are substantially reduced or lost in the vast majority of Pca. In contrast to uniform 15-LOX-2 immunostaining in benign glands, tumors were focally (> 25 %) and completely 15-LOX-2 negative in 59/97 (61 %) and 41/97 (42 %), respectively. In paired tissues from the same patients, 15-HETE formation and 15-LOX-2 mRNA were reduced in tumor vs. benign in 12/14 and 10/11, respectively. In contrast, significant changes in 5-HETE and 12-HETE formation have not been detected. Increased COX-2 mRNA was detected in 3/7 tumors vs. benign. 15-HETE may be a ligand for peroxisome proliferator activated receptors (PPARs), particularly PPARγ. By RT-PCR, we have detected mRNA for PPARα, β, and γ in 18/18 benign and in 9/9 tumor specimens. 15-HETE induced PPARγ-dependent transcription in Pca cell lines. 15-HETE/ PPARγ signalling effects on cell proliferation and differentiation are being examined. Studies in wild type and the Tag transgenic mouse model of Pca have not detected the 8-LOX murine homologue of 15-LOX-2. The major AA metabolite in the Tag mouse is 12-HETE. Reduced 15-LOX-2 is the major alteration in AA metabolism in human Pca. This may contribute to the malignant phenotype by reduced PPARγ activation. The Tag mouse will be a useful model for delineating the contribution of certain AA pathways to Pca.

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INTRODUCTION

Arachidonic acid (AA) metabolism in prostate adenocarcinoma (Pca): AA metabolism may be important in the development or progression of Pca. Recently, Brash et al. discovered a second 15S-lipoxygenase (15-LOX-2), with only approximately 40 % sequence identity to previously characterized human lipoxygenases, including the reticulocyte type of 15-LOX (15-LOX-1) (1). 15-LOX-2 mRNA was detected in a limited number of tissues, including prostate, lung, and cornea (1). A major goal of this proposal was to characterize the expression of 15-LOX-2 in benign prostate and its possible reduction in Pca. As described herein, we have recently demonstrated that benign prostate uniformly expresses 15-LOX-2 and produces 15-HETE from exogenous AA (2) (Appendix 2). In contrast, 15-LOX-2 immunostaining and 15-HETE production from AA is substantially reduced or absent in Pca (2). We have expanded these observations by successfully demonstrating the reduced expression of 15-LOX-2 mRNA in Pca (details in Body). Alterations in 12-LOX mRNA have been described in a significant percentage of PCas (3). 12-HETE in carcinoma cell lines may contribute to tumor growth and angiogenesis (4), invasion and metastasis (5-7). 12-HETE production by actual prostate tissues has not been examined. Alterations in 5-LOX and cyclooxygenase-2 (COX-2) have been suggested from studies in PCa cell lines (8-11). Others have detected increased prostaglandin E_2 in incubations of PCa vs. BPH tissues (12). Hence, complex changes in AA metabolism may accompany Pca development and progression. Although primarily directed at 15-LOX-2, studies described herein have provided more direct insight into possible 12-HETE amd 5-HETE production and COX-2 expression in actual prostate tumors (vs. cell lines).

Peroxisome proliferator activated receptors (PPARs) and Pca: PPARs are a family of nuclear transcription regulating molecules related to other members of the steroid nuclear receptor supergene family (13). Three different species of PPAR have been defined (α, β, and γ), with different organ distributions, ligands, and presumed biological function (13-15). Troglitazaone, a selective PPARγ agonist used in diabetic patients with insulin resistance (16), has been shown to inhibit proliferation and induce differentiation of breast and colon carcinoma cell lines (17, 18). Recently, certain AA metabolites such as prostaglandin J_2 and 8S-HETE, have been demonstrated to activate PPAR-dependent transcription (19-22). In our studies, we have hypothesized that 15-HETE functions in regulation of prostate cellular differentiation, that this is mediated via PPAR activation, and that loss of this function is associated with development or progression of a malignant phenotype. This is a particularly intriguing hypothesis in light of evidence that synthetic activators of PPAR inhibit Pca cell line growth *in vitro* and show activity against hormone insensitive prostate cancer *in vivo* (23-26). In recent unrelated studies regarding oxidized low density lipoprotein (LDL), Nagy et al. demonstrated the activation of PPARγ-dependent transcription by a variety of oxidized lipids, including specifically 15-HETE (27). These studies support our hypothesis of a possible role of reduced 15-HETE activation of PPARγ activation in prostate carcinogenesis or progression. Our studies addressing this hypothesis are described herein.

AA metabolism in transgenic mouse models of Pca: Adequate animal models are essential for delineating the molecular alterations reponsible for tumor development and progression and will allow for testing specific treatment interventions. p53 mutations are likely an important alteration in Pca (28-32). Tag mice created in the laboratory of Dr. Robert Matusik with a prostate epithelial specific long probasin promoter linked to the SV-40 large T antigen (which binds p53) develop precursor lesions, analagous to human high grade prostatic intraepithelial neoplasia (HGPIN), and invasive carcinoma (33). In order to further characterize contributions of altered AA metabolism to tumor growth, we have begun characterizing possible expression of 8-LOX (murine homoloque of 15-LOX-2) and AA metabolism in the Tag mouse. An exciting development includes castration studies in the 12T-7f Tag line, which have demonstated tumor outgrowth, providing a model of androgen independent advanced cancer (34). Another line, 12T-10, consistently devlops a lesion compatible with HGPIN, invasive carcinoma, and metastases, and will be a major focus for future investigations. Studies examining possible alterations in AA metabolism in the different LPB-Tag lines are described herein.

RESEARCH ACCOMPLISHMENT-STATEMENT OF WORK

I. To define the role of 15-LOX-2 in the evolution of human prostate cancer by examining the expression of 15-LOX-2 and the production of 15S-HETE and other AA metabolites in normal, premalignant, and malignant prostate tissue.

A) Using frozen tissue from prostatectomy specimens from the Vanderbilt Urology/Pathology and the Baylor College of Medicine Pathology archives, perform 15-LOX-2 enzyme assays (HPLC analysis of AA metabolites) on paired normal and malignant tissues from a total of 30 patients, 10 each with well, moderately, and poorly differentiated carcinomas. (months 1-12).

Research accomplishment:

Basic methodology and tissue acquisition: We have successfully developed and employed enzyme assays to investigate the production of 15S-HETE and other AA metabolites in prostate tissues. Tissue incubations are typically 45 min. The assay methodology generally employed has been reported by us recently in the attached reprint (2) (Appendix 2). It utilizes addition of exogenous ^{14C}[AA] to homogenates of prostate tissues, which have been rigorously histologically confirmed as to benign, malignant, or mixed. Extracts are analyzed by reverse phase HPLC with in line radiodetection and addition of non-radiolabelled standards to precisely monitor retention times. We initially utilized fresh (not snap) frozen tissues that had been procurred from radical prostatectomy (RP) specimens by the Deptartment of Pathology and the Human Tissue Acquisition and Shared Resource Core of the Vanderbilt Ingram Cancer Center, Vanderbilt University Medical Center. We have found that enzyme activity is preserved in these tissues, despite concerns over RNA degradation (see below). These analyses originally included 8 benign prostate samples (included in first manuscipt, appendix 2) and four pairs of pure benign and pure cancer tissue from the same patients, three of which were included in the first manuscript (2) (Appendix 2). Again as reported in the attached recent American Journal of Pathology paper (Appendix 2), we have found that benign prostate uniformly synthesizes 15S-HETE from exogenous AA and that 15-HETE is the major metabolite formed under our incubation conditions (see Figure 1, Appendix 2). The identity of this product was established by retention times on reverse phase HPLC, its characteristic UV spectrum, and its retention time on straight phase HPLC in a subset of samples. In contrast to benign prostate, 15-HETE production is substantially reduced or absent in incubations of prostate carcinoma (Pca) tissue (2) (see Figure 3, Appendix 2). We have now extended these studies and broadened the scope to more specifically address formation of other lipoxygenase (LOX) and cyclooxygenase (COX) products utilizing snap frozen prostate tissues obtained in collaboration with Baylor College of Medicine, Houston, TX as outlined in the original grant proposal.

We have enjoyed a tremendously productive interaction with our colleagues at Baylor who have an extensive data base of snap frozen prostate tissues generated as part of a Prostate SPORE grant. These tissues are obtained in the operating room by 6 mm punch biopsies from multiple sites within the prostate, yielding cores approximately 1 to 1.5 cm in length (35). These tissues are processed by us for RNA and enzyme assays as shown in Figure 1 of Appendix 1, with careful correlation to the specific histology of the tissue actually processed for these analyses. Histologic examination includes percent benign, percent malignant, grade of tumor, percent glands and stroma, and any unique histologic features (e.g., atrophy of benign glands). We have thus far processed 55 snap frozen potential pairs of benign and malignant tissue from the same RP specimens. Histologic assessment of portions submitted for RNA and AA incubations has yielded a substantial number of samples adequate for examining potential differences in gene expression and AA metabolism between benign prostate and prostate adenocarcinoma (Table 1). Initial aims included investigation of different grades of tumor, designated "well, moderately, and poorly differentiated" in the original proposal. This designation usually translates to Gleason grades 2-4, 5-7, and

8-10. Gleason grade 2-4 tumors are generally small transition zone tumors, which may be discovered incidentally in TURP procedures, in autopsy prostates, or in radical prostatectomies performed for higher grade index tumors in the peripheral zone (PZ). A number of such Gleason pattern 2 tumors were included in our original report on 15-LOX-2 in benign and malignant prostate (2). Such small TZ tumors detected clinically do not necessarily warrant definitive treatment (e.g., Stage T1a tumors in TURP). In additon, it is clear that Gleason 7 tumors behave more aggressively than Gleason 5-6 tumors (36). Additionally, as most of the radical prostatectomies demonstrate index tumors in the peripheral zone (PZ) and have been performed for clinical stage T1c or T2 tumors, we will investigate possible correlation of altered AA metabolism with tumor grade (and potentially outcome and other pertinent clinical and pathologic parameters) by dividing tumors into those of Gleason grade categories of 5-6, 7, and 8-10, paralleling increasing clinical severity and biologic aggressiveness of Pca (36). Hence, we have thus far concentrated our analyzes on PZ tumors and benign tissue counterparts from the PZ. Intraoperative biopsies are also taken from the TZ (35) and some of these may have been clinicallyl detected or demonstrate tumor in subsequent RP surgical pathology analysis as incidental lesions. Based on the results of our investigations, we may ultimately want to examine some such tumors as well.

Our processing of these snap frozen tissue cores has also confirmed the difficulty of obtaining pure benign and pure tumor tissues for studying differences in gene expression (Table 1). This is expected given the known property of prostate carcinoma to infiltrate amongst benign glands, an aspect of prostate carcinoma making it a unique candidate for study by laser capture microdissection (LCM) (37-39), which we have begun incorporating into our investigations (see below)..

TABLE 1

Histologic classification of paired snap frozen tissues *	n
Benign + Benign	11
Benign + Mixed	17
Mixed + Mixed	4
Benign + Tumor	18
Mixed + Tumor	1
Tumor + Tumor	2
Other (including seminal vesicle)	2

* Benign = > 85 % benign, Tumor = > 85 % tumor, mixed = all other combinations

The preparation of a formalin fixed paraffin embedded (FFPE) section from tissue between that processed for RNA and AA incubation not only allows for histologic documentation, but also provides material for immunohistochemistry. Even more importantly, these tissue sections as well as the OCT embedded frozen section (See Figure 1, Appendix 1) also give us a valuable pool of material for comparing frozen and fixed sections for LCM coupled to RT-PCR for analysis of gene expression in benign and malignant prostate, as these snap frozen cores have optimal RNA preservation. Such LCM based studies may represent a major focus of future research efforts and potential grant applications.

Arachidonic acid metabolism in snap frozen prostate tissues: Utilizing ^{14C}[AA] incubations and RP-HPLC analysis similar to those reported by us previously (2) (Appendix 2), we have detected 15-HETE formation in 22/27 incubations of benign prostate from 24 patients. 15-HETE formation has also been detected in incubations of 6/6 samples of mixed benign and tumor (reflecting either the contribution of benign glands or subtotal loss of activity in cancer portions), and only 1/4 tumors (in which benign tissue was not available from the same patient). In two negative benign samples, there was transitional metaplasia and atrophy, known to be negative for 15-LOX-2 based on our published immunohistochemical

results (2) (Appendix 2). In pairs of pure benign and Pca snap frozen from the same patient, 15-HETE production from AA was reduced in Pca compared to benign in 8/10 such samples analyzed thus far.

Although focus has been placed on the production of 15S-HETE by benign prostate and possible reduced 15-HETE in prostate adenocarcinoma, a major advantage of the methodology employed by our laboratory is the detection of other AA metabolites. Studies by others have indicated possible upregulation of platelet type 12-LOX mRNA in Pca, particularly high grade, high stage tumors (3). Studies in prostate cancer cell lines have indicated the possible presence of 5-LOX in PC-3 cells (8) and COX-2 in PC-3 and LNCaP cells (10, 11). Furthermore, studies showing inhibition of proliferation and induction of apoptosis with 5-LOX, FLAP, and COX-2 inhibitors have suggested a possible role for these enzymes in regulation of cancer cell growth (9, 11). Studies *in vitro* and in nude mice using PC-3 cells stably transfected with 12-LOX have suggested a possible role of 12-HETE in tumor angiogenesis (4). However, to our knowledge no one has reported 12-HETE formation in prostate or possible increased 12-HETE formation in prostate cancer and no one has reported possible alterations in 5-LOX or COX-2 in actual prostate cancer tissues. We have begun addressing these issues in more systematic fashion.

Our usual RP-HPLC assay conditions involve injection of one-fifth of the incubated sample, a solvent system of methanol/water/glacial acetic acid at 80:20:0.01 (by vol.), a flow rate of 1.1 ml/min. and in line radiodetection. This results in a retention time for 15-HETE of approximately 15 min. and adequate resolution of the other HETEs (with simultaneous injection of unlabeled 5-, 8-, 9-, 11-, 12-, and 15-HETE) (2) (Appendix 2). Although possible prostaglandin production may be suggested by the presence of more polar metabolites eluting early in the HPLC run, this solvent system does not allow for adequate separation of potential COX-2 metabolites, such as PGE_2, from other prostaglandins or other possible polar products not derived from COX metabolism. We have not routinely detected any 5-HETE, 12-HETE, or possible PG peaks in incubations of benign prostate as routinely performed (described in Appendix 2). As most data suggesting a potential role for these metabolites has been generated in cancer cell lines (particularly androgen insensitive and highly karyotypically abnormal PC-3 cells), we have focused attention on possible production of these metabolites primarily in tumor incubations, with inclusion of a number of high grade tumors (Gleason grade $\geq$ 7). Under conditions in which 15-HETE is readily detectable in benign prostate, neither 5-HETE nor 12-HETE production were detected in any of 19 tumor AA incubations (6 mixed benign and tumor, 13 pure tumor). In order to be more certain that we have not missed possible low levels of 5-HETE or 12-HETE formation and possible differences between benign and malignant prostate, we have analyzed a greater sample volume (four-fifths of the incubation extracts) using a more prolonged HPLC solvent system in a subset of tumors and benign-tumor pairs. In three pairs of benign and malignant tissue (all Gleason grade 8 tumors) this incorporated a solvent system of methanol/water/glacial acetic acid at 77.5:22.5:0.01 (by vol.), a flow rate of 1.0 ml/min, and collection of 0.5 min fractions and 10 min. scintillation counting to increase the signal to noise ratio. Very trace 5-HETE formation (< 5 % of amount of 15-HETE formed) was present in one benign sample. Neither 5-HETE or 12-HETE was detected in tumor incubations and certainly there was no indication that there may be increased 5- or 12-HETE production in tumor vs. benign (not shown).

In another set of samples, four-fifths of the incubation extract sample was again injected, this time using a solvent system designed for optimal resolution of prostaglandins, while still allowing for resolution of the individual HETEs. This system employs a solvent system of acetonitrile/water/glacial acetic acid (35:65:0.01, by vol.) 0-20 min., acetonitrile/water/glacial acetic acid (60:40:0.01, by vol.) 20-40 min., 100 % methanol 40-60 min. at 1.1 ml/min, with coinjection of cold TxB_2, 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, PGD_2, HHT, 15-HETE, 11-HETE, 8-HETE, 12-HETE, 9-HETE, and 5-HETE and in-line radiodetection. This analysis was extended to two benign and tumor pairs (Gleason grade 7 and 8) and three additional tumors (Gleason grade 5, 6, 8). Very trace (significantly less than amounts of 15-HETE formed in benign and even some malignant samples) 5-HETE was detected in two of the separate tumor samples and one benign sample of a benign-tumor pair; very trace possible 12-HETE was detected in two of the separate tumor

samples and one benign sample of a benign-tumor pair. In order to be certain that our incubation conditions were adequate to detect 5-LOX or 12-LOX activity if present, we have spiked prostate homogenates previously determined to be negative for 5- and 12-HETE formation with leukocyte extracts as a source of 5-LOX and purified recombinant human 12-LOX. 5-HETE and 12-HETE were readily detected in 45 min. incubations of these respective preparations in buffer alone, with > 75 % equivalent peak heights in similarly spiked prostate homogenates (not shown). These results indicate that incubation conditions are adequate to detect 5-LOX and 12-LOX. Clearly, based on results of 15 analyses of benign and malignant pairs from the same patient, 5 of which included an increased sample volume, we do not detect increased 5-HETE and/or 12-HETE in tumor vs. benign prostate tissue and formation of these LOX metabolites does not appear to be a feature of AA metabolism in Pca.

Regarding possible COX (COX-2) metabolites in these expanded HPLC analyses, very trace possible PGE_2 and HHT formation was detected in two of the three separate tumors, in one tumor of a benign-tumor (Gleason 7) pair and in the benign of a separate benign-tumor (Gleason 9) pair. Although altered PG production thus does not appear to be a characteristic alteration in Pca, the possible expression of COX-2 in Pca has been addressed in a higher number of samples at the mRNA level as described below.

The results of the studies on AA metabolism in benign and malignant prostate utilizing snap frozen tissues, combined with the results of 15-LOX-2 Northern blots and COX-2 protection assays described below, are being submitted in abstract form to the 2000 meeting of the American Urological Association in Atlanta, GA.

B) Using paraffin embedded sections from these same 30 prostatectomies, perform immunohistochemical staining for 15-LOX-2. Additional whole mount sections will be utilized if necessary to examine adequate numbers of low and high grade PIN lesions in assocation with carcinomas. (months 1-12).

Research Accomplishments: We have used immunohistochemistry on paraffin embedded sections from radical prostatectomies (RP) to characterize the expression of 15-LOX-2 in benign prostate and its possible reduction in Pca. We have used a polyclonal antiserum prepared against purified human 15-LOX-2, generally at a 1:2500 dilution, and a Ventanna auto-immunostainer, as detailed in the attached reprint (2) (Appendix 2). We have demonstrated that this antibody is specific for human 15-LOX-2 (and the murine homologue, 8-LOX, described below) and does not cross react on western blots with human platelet type 12-LOX, 5-LOX, or 15-LOX-1. 15-LOX-2 is uniformly present in apical or secretory cells of the PZ and secretory cells of prostatic ducts, and is also present (with less intense and less uniform immunostaining) in secretory cells of the TZ and central zone (CZ). It is not present in basal cells, prostatic stroma, transitional epithelium, ejaculatory ducts, or seminal vesicles (2) (See Table 1 and Figure 2 of Appendix 2). 15-LOX-2 immunostaining is reduced to a variable degree in benign atrophic PZ glands, generally paralleling the degree of atrophy (See Figure 2, Appendix 2). In our original report, we characterized possible reduced immunostaining of 15-LOX-2 in Pca utilizing 18 RP cases, three of which matched the samples analyzed by HPLC and an additional 15 cases to reflect varying tumor grades and stages (2). In contrast to the uniform immunostaining for 15-LOX-2 in benign prostate, 15-LOX-2 was reduced to a variable extent in the majority of Pcas. 14 of 18 cases showed loss of 15-LOX-2 in > 25 % of the tumor (mean 74.9 % negative for 15-LOX-2; range 38.9 – 100 %) and 10/18 cases showed essentially complete loss of 15-LOX-2 immunostaining (2) (See figure 4, Appendix 2). In this small number of cases, we did not examine correlation with grade. However, in individual cases, there was evidence of correlation with grade, including retained 15-LOX-2 immunostaining in Gleason pattern 2 with loss in Gleason pattern 3 in one case, retained 15-LOX-2 in Gleason pattern 2, with partial loss in Gleason pattern 3, and loss in Gleason pattern 4 in a second case, and retained immunostaining in acinar Gleason pattern 3 and loss in ductal carcinoma in a third case (See Appendix 2, including figure 4). In tumors with partial

loss of 15-LOX-2, there was marked heterogeneity of immunostaining within different tumor foci (2) (See figure 5, Appendix 2).

We have now expanded these studies to undertake a more rigorous examination of possible correlation of 15-LOX-2 expression with tumor grade and other possible pathologic and clinical parameters, including patient outcome. Although we originally proposed to possibly utilize the same cases/tissues processed for enzyme assays with supplementation with RP whole mount sections as indicated, because of possible heterogeneity of 15-LOX-2 expression in cancer tissue and the increased ability to correlate staining with precise histologic features (tumor gland architecture, sites of capsule penetration, associated PIN, etc), we have primarily performed these immunostains on sections taken from formalin-fixed paraffin embedded (FFPE) whole mount sections. As part of my clinical responsibilities and paralleling my interest in genitourinary pathology, I sign out the surgical pathology of all the radical prostatectomy specimens at Vanderbilt (beginning Jan. 1998). I have signed out approximately 200 totally submitted whole mount embedded RP specimens over the past two years. This has allowed for the creation of a data base with uniform assessment of pathologic parameters, such as grade, stage, and margin status, as well as tumor volume determinations by digital planimetry. This has greatly facilitated our studies on AA metabolism in Pca as well as collaborative clinicopathologic projects with members of the Department of Urology. Greg Jack, a Vanderbilt medical student predoctoral fellow spending a year in my laboratory, in collaboration with Sandy Olson in the Department of Pathology Research Immunohistochemistry Lab, has recently completed immunostaining on 60 RP specimens (20 Gleason grade 5-6, 20 Gleason 7, 20 Gleason 8-10, 10 each with associated HGPIN). The results of these studies are currently being analyzed.

In addition, in an initial effort to correlate reduced 15-LOX-2 expression in Pca with patient outcome, in collaboration with Drs. Thomas Wheeler and Peter Scardino at Baylor College of Medicine and The Methodist Hospital, Houston, TX, we performed 15-LOX-2 immunostaining on sections of tumor from 50 cases without recurrence of Pca (9-15 years follow-up) and 50 cases with recurrence. These stains were done on 6 mm cross sections retrieved from the RP paraffin blocks, based on tumor location in matching H & E stained slides. Because of smaller size of these sections, the tumor portions on the immunostained slides were assessed for absent 15-LOX-2 semi-quantitatively, as follows: 0 = uniformly 15-LOX-2 positive; 1 = 0 – 25 % 15-LOX-2 negative; 2 = 25 – 50 % 15-LOX-2 negative; 3 = 50 – 75 % 15-LOX-2 negative; 4 = > 75 % 15-LOX-2 negative; 5 = 100 % 15-LOX-2 negative. Results of this study showed that overall, a similar percentage of prostate cancers lost 15-LOX-2 expression as in our studies on tissues processed at Vanderbilt, extending our observations to another data set. Althouth there was no apparent difference between the patients without and with recurrence (Table 2), we have not yet analyzed these with respect to grade, stage, and PSA. We anticipate a more complete study of this nature on material from RPs at Vanderbilt, with our data base as described above or similarly analyzed older cases, to allow for longer follow-up.

Table 2 –15-LOX-2 Immunostaining on Cases from The Methodist Hospital, Houston, TX

	Non-recurrent N = 37*	**Recurrent N= 42***
Completely neg.	15/37 (41 %)	16/42 (38 %)
> 75 % neg.	19/37 (51 %)	19/42 (45 %)
> 25 % neg.	21/37 (57 %)	24/42 (57 %)

*Number with adequate amount of tumor on slides stained

C) Northern blots or RT-PCR and western blots for 15-LOX-2 on mRNA and protein from frozen normal and malignant tissue from prostatectomy specimens containing well, moderately, and poorly differentiated carcinoma. Where possible, the same specimens as in part A will be employed. Sufficient numbers of additional archival cases will be processed if necessary. This material will be utilized in completion of portions of task 2 as well. (months 6-15).

Research Accomplishments:

15-LOX-2 Western and Northern blots: Western blots and Northern blots have in general confirmed our results with immunostaining and enzyme assays, demonstrating the presence of 15-LOX-2 in benign prostate and reduced expression in Pca. To examine possible loss of 15-LOX-2 at the transcription level, we have placed more emphasis on Northern blots for examination of benign vs. cancer. 15-LOX-2 protein by Western blot and 15-LOX-2 mRNA by Northern blot were detected in 8/9 and 19/21 benign samples, respectively (not shown). Suzanne Manning, a molecular biologist in my laboratory, hired and supported by this grant, now performed over 20 northern blots for 15-LOX-2 in prostate. We have markedly improved our techniques, including the use of a Packard Phosphorimager, now achieving quantitative results in 6 hr. to overnight exposures, normalizing to housekeeping genes (generally GAPDH) for comparison of 15-LOX-2 mRNA in benign vs. malignant tissue. In pairs of pure benign and Pca from the same patient, we have demonstrated that 15-LOX-2 mRNA is reduced in Pca compared to benign in 10/11 patients (See Figure 2, Appendix 1).

Preliminary COX-2 mRNA analyses: Few data exist regarding possible altered prostaglandin production in Pca. Using a thin layer chromatography assay, Chaudry et al. reported increased PGE_2 formation from AA in malignant prostate tissue compared to BPH (12). The cells responsible or the enzymatic source (e.g., COX-1 vs. COX-2) have not been addressed to our knowledge. As described above, others have reported the expression of COX-2 mRNA in PC-3 cells (10) and LNCaP cells, and induction of apoptosis of LNCaP cells by very high levels of selective COX-2 inhibitors (11). Prostaglandin (PG) production or possible correlation of anti-proliferative effects of COX-2 inhibitors with inhibition of PG formation were not addressed.

In contrast to epidemiologic evidence suggesting decreased incidence of colorectal carcinoma with NSAID usage and data suggesting increased COX-2 in colorectal carcinoma, limited epidemiologic studies thus far do not support a reduction in prostate carcinoma with NSAID usage (40), although these agents may not penetrate prostate tissues adequately. In addition, to our knowledge, no one has yet reported the increased expression of COX-2 in actual Pca tissues (vs. cell lines). As detailed above, we have not consistently observed increased PG production in Pca tissues using standard incubation conditions employed for 15-HETE determination. However, in contrast to studies with 5-HETE and 12-HETE production, we have not yet rigorously determined that our incubation conditions are optimal for detecting COX-1 or COX-2 activity and PG accumulation. In a manner similar to use of exogenous 5-LOX and 12-LOX as described above, we are currently addressing this issue using purified, enzymatically active recombinant human COX-2 (provided by Dr. Larry Marnett, Dept. of Biochemistry, Vanderbilt Univ. Medical Center). We are also supporting these enzyme assay studies by examining COX-2 mRNA in Pca. We have recently collaborated with Dr. Matthew Breyer on a study utilizing bladder biopsy and cystectomy specimens and in situ hybridization and immunohistochemistry which demonstrated increased expression of COX-2 in high grade urothelial carcinomas (manuscript in preparation). Rnase protection assays demonstrated COX-2 mRNA in some bladder cancer cell lines. We have taken advantage of the expertise of Dr. Breyer's laboratory to corroborate our PG assays by examining possible COX-2 mRNA in Pca tissues (snap frozen cores as utilized for 15-LOX-2 Northern blots). Again, because if Pca cell lines are similar to any actual prostate cancer tissues, it is likely to be high grade, high stage tumors (as these

cell lines were established from advanced or metastatic tumors, in addition to being propagated in vitro). Hence, we have focused particular attention on high grade tumors thus far. By RNase protection assays, COX-2 mRNA was negative in benign and tumor in four pairs from the same patients, positive in tumor and negative in benign in another pair, and faintly positive in two additional tumors (3 of 7 total tumors analyzed weakly or strongly positive). No message was detected in 6 additional benign and 7 additional tumors, but controls were suboptimal in these analyses, so they are being repeated and more cases are being analyzed. In two of the three with possible positive COX-2 message, therewas trace PGE_2 and HHT formation. No possible prostaglandin products were detected in incubations of parallel tissue portions in the third tumor sample (not shown). It appears that COX-2 mRNA may be upregulated in some Pca. In what cell types COX-2 message is expressed and whether it translates to enzymatically active protein remains to be more fully examined.

D) 15-LOX2 immunohistochemistry on paraffin embedded tissue on prostate biopsies containing different grades of prostate cancer and PIN (10 each of normal, low grade PIN, high grade PIN, cancer with low grade PIN, cancer with high grade PIN) (months 9-18)

Research Accomplishments and Future Aims: As expected from the results of studies in radical prostatectomy specimens, 15-LOX-2 immunostaining on routinely processed formalin-fixed paraffin-embedded prostate needle core biopsies shows strong staining of benign glands, with variable loss of immunostaining in prostate adenocarcinoma (Figure 3a,b, Appendix 1). These results demonstrate the validity of biopsy immunostaining for studying expression of 15-LOX-2 within both benign and malignant glands in biopsy materials. In addition to a simple correlation with grade and possible association of absent 15-LOX-2 in invasive carcinoma with normal or reduced expression in HGPIN (as possible indication of a late or an early change in cancer developement, respectively), we are in the process of compiling a case data base for a more complete retrospective study. This will include loss of 15-LOX-2 on biopsy, in addition to grade, extent of biopsy involvement with cancer, and serum PSA, correlated with findings in the radical prostatectomy specimen (including stage, tumor volume, and margin status). Similar to other examined potential prognostic factors, a desirable outcome is the further strengthing of preoperative predictions of which patients may have non organ confined disease. In addition, correlating 15-LOX-2 immunostaining of Gleason 6 tumors on biopsy with tumor volume in RP specimens may help define patients with clinically insignificant disease who may be safely treated conservatively. As with any biopsy based study, concern exists over adequacy of sampling (whether tumor in the biopsy is representative of the tumor in the RP specimen), potentially compounded by the heterogeneous nature of reduced 15-LOX-2 expression in Pca (as demonstrated thus far in RP specimens). The number of cases immunostained and examined thus far is too small to allow for any meaningful data analysis (project intended primarily for year 2 of grant perioid). The compilation of cases for our ongoing studies correlating free PSA with RP path parameters (now being extended to biopsy findings) will facilitate these future studies on 15-LOX-2 in biopsies.

II. To establish the presence of PPAR subtypes in benign and malignant human prostate and evaluate the activity of 15-HETE in activation of these PPAR subtypes.

A) Northern blots or PCR for PPAR subtypes in paired benign and malignant tissue from archival prostatectomy samples. In general, the same specimens as in Task 1, part C above will be utilized. If necessary, additional specimens will be processed. (months 9-18).

Research Accomplisments: We originally hypothesized that 15S-HETE may be capable of activating PPAR and PPAR-regulated gene transcription. This was based in part on previous

demonstrations that AA metabolites, including prostaglandins of the J series and 8S-HETE, may activate PPAR (19-22). Since our original grant submission, in addition to our own experiments, two pieces of data have emerged from other laboratories which strongly support our original hypothesis and which have led us initially to focus more attention on PPARγ. In investigations related to atherogenesis, oxidized LDL was shown to activate PPARγ in macrophages and in parallel experiments examining specific oxidized lipids, 15-HETE (in addition to 9-HODE and 13-HODE) activated PPARγ-dependent transcription *in vitro* (27). Regarding PPARγ and Pca, Kubota et al. have recently shown that PPARγ is constituitively expressed in PC-3, LNCaP, and DU-145 prostate cancer cell lines and that PPARγ-agonists, including the thiazolidinedione troglitazone and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, inhibit proliferation of PC-3 cells *in vitro* and in nude mice (26). Little data was presented in actual prostate tissues, although these authors showed a Western blot demonstrating PPARγ protein in extracts of a case of Pca and indicated a possible increase compared to benign (26).

We have begun characterizing the possible expression of PPAR species in benign and malignant prostate, the possible role of 15-HETE in regulating PPAR activity in prostate, and possible alterations of this signaling mechanism is Pca. RNA extracted from snap frozen cores of prostate tissue determined to be benign or malignant as demonstrated in Figure 1 and described above for 15-LOX-2 experiments was used to examine the presence of mRNA for the three different defined subtypes of PPAR (α, β or δ, and γ). By RT-PCR, we detected message for all three PPAR subtypes in 18/18 benign prostate specimens and in 9/9 pure prostate tumor specimens (unpublished observations, not shown). Because of data demonstrating inhibition of proliferation of PC-3 cells by the synthetic PPARγ agonists troglitazone and BRL 49653 and PGJ_2 (26) and the possible activation of PPARγ by 15-HETE (27), we have attempted to further characterize the expression of PPARγ in prostate and possible differences between benign and malignant. These experiments have been paralled by *in vitro* studies examining possible activation of PPARγ by 15-HETE in Pca cell lines and possible effects of 15-HETE vs. known PPARγ agonists on growth and differentiation of Pca cell lines (described below).

Initial efforts have included in situ hybridization for PPARγ. Formalin fixed paraffin embedded tissues from the snap frozen cores (See Figure 1, Appendix 1) provide an ideal tissue source for in situ hybridization, with likely superior preservation of RNA (e.g., compared to conventionally preserved surgical pathology material). Furthermore, although not quantitative, as this approach allows for correlation of expression with histology, it can be used to examine PPARγ in benign and malignant prostate even in cores with admixed tumor and benign glands (See Table 1). We have collaborated in this endeavor with Dr. Breyer's laboratory, which has previous experience with PPARγ in situ hybridization, including in examination of bladder carcinoma (41). However, thus far by this technique, we have been unable to detect expression of PPARγ in benign or malignant prostate in any of 24 sections examined (with good controls) (not shown).

Although quantitating possible differences in PPARγ gene expression (vs. identifying patterns of expression) in benign vs. malignant prostate was not a specific component of the proposal, we have attempted to develop quantitative assays for PPARγ expression given the emerging evidence that 15-HETE may be an endogenous ligand for PPARγ in prostate and the suggestion by Kubota et al. (26), based on a small number of cases, that PPARγ protein may be increased in Pca. We have attempted to measure levels of PPARγ mRNA in RNA extracted from benign and malignant prostate cores by both Northern blots and, in collaboration with Dr. Breyer's laboratory, by Rnase protection assays. We have been unable to detect PPARγ mRNA in any of 24 benign or malignant prostate samples by either of these techniques, again with good controls (not shown). The results showing uniform presence of PPARγ mRNA in prostate by RT-PCR, but lack of detection by in situ hybridization, Northern blots, or protection assays would indicate to us that the levels of mRNA for this gene must be extremely low in the human prostate. This issue, in addition to our desire to quantitate levels of gene expression in RNA derived from laser capture microdissection (LCM), has led us to explore the development of quantitative real time PCR assays (42-

44). We have recently facilitated the purchase of a Roche LightCycler Real Time PCR instrument by Dr. DuBois' laboratory. In our brief recent experience with this instrument, we were able to achieve linear PCR results (based on dilutions of template) with our PPARγ primers and a fluorescent probe which binds to double stranded DNA (SYBR green) and linear results with PCR for the housekeeping gene β-actin using specific hydrolysis (TaqMan) probes (44); (data not shown). A long term commitment to this powerful technology, both for quantitating RNA from tissues procured as described here (Figure 1, Appendix 1) and LCM derived cells (44), has prompted us to seek separate funding for the further development and application of this technique for analyzing gene expression in Pca (including aCulpeper Foundation Medical Scholarship, for which Dr. Shappell was the 1999 Vanderbilt nominee).

In addition to attempts to measure PPARγ mRNA in actual prostate tissues, we have developed immunohistochemical techniques for PPARγ in formalin fixed paraffin embedded tissue. Immunohistochemical asssays for PPARγ have apparently been difficult to develop with existing antibodies, and there are few reports in the literature, with illustrated examples of acceptable quality immunostains (including breast carcinoma (17) and atherosclerotic lesions (45). Immunohistochemical staining for PPARγ in prostate was commented on, but no results were shown by Kubota et al. (26). Thus far, the best results we have achieved have utilized a commercially available monoclonal antibody to PPARγ, clone E8 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). A primary antibody dilution of 1:10 is used in overnight incubations at 4 °C. These conditions were determined to be optimal. Antigen retrieval has not improved results (not shown). Bladder cancer sections (including from some cases previously shown by Dr. Breyer's lab to express PPARγ message by in situ hybridization and a subset of breast cancer cases (17) are used as positive controls (Figure 3c, Appendix 1). Thus far, we have performed immunohistochemical studies on the 17 tumor and 12 benign sections from radical prostatectomy specimens and the sections from the 100 cases with long term follow-up obtained in collaboration with Dr. Thomas Wheeler, Baylor College of Medicine, Houston, TX, and previously immunostained for 15-LOX-2 as described above. Very few cases (< 5 %) have thus far demonstrated convincing true nuclear staining in a small percentage of tumor cells (Figure 3d, Appendix 1). Interestingly, one case with cribriform Gleason pattern 4 tumor that showed true nuclear staining for PPAR γ had been observed to show very strong 15-LOX-2 immunostaining. It remains our hypothesis that PPAR γ is normally expressed in prostate (although potentially at low levels), that 15-HETE is an endogenous ligand for PPARγ in regulation of prostate cell differentiation, and that reduced 15-LOX-2 and 15-HETE in Pca contributes to the malignant phenotype by a reduction in this signalling mechanism. As detailed above, more sensitive techniques for assessing PPARs in prostate tissue will be required. However, in light of very recent evidence of PPARγ mutations in some colon cancers (46), it is interesting to speculate that such mutant forms may accumulate and be more readily detectable by immunostaining (analagous to p53 mutations). Such mutations may result in compensatory increases in 15-LOX-2, accounting for the marked expression in a very minor subset of tumors. We plan to explore this, perhaps utilizing LCM coupled to DNA extraction and PCR (which we have been able to successfully achieve for other applications in our laboratory), initially utilizing primers designed for regions shown to be mutated in colon cancer (46).

B) Examination of 15S-HETE vs. other AA metabolites to activate PPAR subtypes identified in part A, using reporter assays available through Dr. DuBois' laboratory (months 18-30).

Research accomplishments: We have already initiated these experiments and are clearly ahead of schedule in this area of the proposal. We have begun to use Pca cell lines to investigate possible activation of PPARγ dependent transcription by 15S-HETE and the possible contribution of 15-HETE and PPARγ to regulation of Pca cell growth and differentiation. Supporting our hypothesis of loss of 15-LOX-2 in Pca, RT-PCR analyses show scant or absent message in three Pca cell lines (PC-3, DU-145, and LNCaP)

(Figure 4a, Appendix 1). Paralleling this lack of 15-LOX-2 mRNA, we do not detect 15-LOX-2 activity in these cell lines (not shown). We have detected constituitive expression of PPARγ in PC-3, DU-145, and LNCaP cells by RT-PCR (Figure 4b, Appendix 1), as also reported by Kobota (26). PPARβ is also constituitively expressed, whereas PPARα is not readily detectable (Figure 4b, Appendix 1).

Working primarily with Rajnish Gupta, an M.D./Ph.D. candidate in Dr. DuBois' laboratory, we have begun to investigate the function of PPARγ in PC-3 and DU-145 cells and the ability of 15-HETE to activate PPARγ-dependent transcription by performing transient transfection assays using a PPARγ-response element cloned upstream of luciferase. This is similar to the experimental approach previously employed in examining the effect of PPARγ agonists on colon cancer cell line proliferation and the ability of these synthetic agents to activate PPARγ-dependent transcription in colon cancer cell lines (18).

PPRE-tk-luciferase is generously provided by Dr. Ron Evans (The Salk Institute, San Diego, CA) to Dr. Raymond DuBois, a consultant on this project. PC-3 and DU-145 cells obtained from the ATCC have been transfected with a mix of 1 ug/ml PPRE-tk-luciferase, 0.05 ug/ml pRL-SV40, and 20 ug/ml Cellfectin in Opti-MEM (Gibco) for 5 hours. The transfection mix was replaced with media with DMSO, 1 or 10 uM of the PPARγ-agonist BRL 49653, or 1 or 10 uM 15S-HETE for 12 hours, followed by harvesting in luciferase lysis buffer. Luciferase activity was assessed with a luminometer and normalized as described (18). 10 uM 15S-HETE caused an approximately 2-fold induction of PPARγ-dependent transcription in both cell lines, lending direct support to our hypothesis that it may function as a ligand for PPARγ in prostate cells and that loss of this activity (by virtue of loss of 15-LOX-2) may be deleterious in prostate cancer cells.

We have begun parallel experiments addressing the possible effect of 15-HETE (vs. known synthetic PPARγ agonists such as BRL 49653) on proliferation and differentiation of prostate cancer cell lines. These experiments expand the scope of our related investigations in the current grant, and we have successfully obtained additional funding for these *in vitro* mechanistic studies (with no specific overlap) in the form of a two year VUMC Intramural Interdisciplinary Discovery Grant (Dr. Shappell, Principal Investigator, $55,000.00/yr, direct). These separate investigations again involve collaboration with the laboratories of Dr. Brash, Dr. Matusik, and Dr. DuBois, an extension of the fruitful collaboration established in this current grant proposal. These experiments are also exploring possible androgen regulation of 15-LOX-2 and possible interactions of 15-LOX-2/PPARγ and androgen signaling in prostate cell lines. These experiments involve cell proliferation assays, ultrastructural investigations of cell differentiation, flow cytometric determinations of effects on cell cycle and apoptosis, and AR modulation of 15-LOX-2 gene transcription and will combine with the results of the current grant to provide tremendous insight into the roles of these pathways in prostate cancer cell regulation.

III. To delineate the role of the murine homologue of 15-LOX-2, a novel 8-lipoxygenase (8-LOX), in prostate cancer development in the LPB-Tag transgenic mouse.

A) Immunohistochemistry for 8-LOX in paraffin embedded prostate tissues of normal mice and LPB-Tag mouse lines 12T-11, 12T-5, and 12T-7(f). 5 mice at each of 2 different time points for each line as indicated in proposal. These time points correspond to times sampled for enzyme assays described below. Additional sections are available at other time points, if necessary (months 3-12).

Research accomplishments: Sequence identity and other structural properties would indicate that a novel 8-lipoxygenase identified in Dr. Brash's laboratory (47) is the murine homoloque of human 15-LOX-2. Despite obvious structural differences between 8-HETE and 15-HETE, data already exists indicating 8S-HETE (but not 8R-HETE) can activate PPARα (19) and more recently, studies by Dr. Susan Fisher have shown results compatible with PPAR activation in transgenic mice overexpressing 8-LOX in skin (Susan Fisher, personal communication). PPAR subtypes present in normal mouse prostate or in the

prostate tumor in Tag mice are not yet known and whether 8-HETE may be a ligand for these specific species remains to be established. Our 15-LOX-2 antibody cross reacts with the murine 8-LOX, and we have used immunohistochemistry (coupled to enzyme assays described below) to begin investigating possible constituitive expression or upregulation of 8-LOX in wild type and Tag mouse prostates. As expected from previous demonstrations of increased 8-HETE and expression of 8-LOX in mouse skin treated with phorbol esters, such skin specimens (formalin fixed and paraffin embedded) have served as positive controls, with strong and consistent immunostain results (not shown). We have performed 8-LOX immunostaining on the dorsolateral prostate of 3-4 12T7-f mice each at 6, 9, 12, 15, 18, and 21 weeks and additional mice at 33 weeks and have not detected any appreciable 8-LOX immunostaining. Given the uniformly negative results, we feel this number of animals is adequate to exclude 8-LOX immunostaining in this Tag line at these time points. In addition, we performed immunostaining on the prostates of 12T-5 mice at 12 and 16 weeks and 12T-11 mice at 12 and 30 weeks and again have not seen 8-LOX immunostaining. 8-LOX immunostaining is also not detected in wild type mice at 7, 10, 13, 17, 18, and 42 weeks (not shown). These specific Tag lines were originally selected as targets of study because they represented different rates of growth of the prostate (33). However, these lesions are characterized histologically by proliferation of cytologically malignant epithelial cells within pre-existing duct and gland structures (analogous to human HGPIN), without unequivocal stromal invasion or metastases. As we are studying true invasive carcinoma in the human (in addition to attention to the precursor lesion HGPIN), these models may not represent a truly analogous situation and do not so far exclude changes in AA metabolism between benign or precursor lesions and frankly invasive tumors. As part of our ongoing collaboration with Dr. Matusik's laboratory, further characterization of Tag mice has demonstrated invasive disease developing in castrated 12T7f mice maintained for subsequent outgrowth of androgen insensitive tumors (34). In addition, the 12T-10 line, not previously completely characterized, shows tremendous promise from a histopathologic point of view. This mouse develops precursor lesions (well documented at 24 weeks), possible microinvasion at 28 weeks, and frankly invasive disease at 44 weeks, at which point metastases are regularly present in lymph node, liver, and lung (unpublished; Masumori, N. et al., manuscript in preparation). In addition, the metastases show cytologic features compatible with neuroendocrine differentiation, which has been confirmed by chromogranin immunohistochemistry and electron microscopy (not shown, manuscript in preparation). This line thus shows many desirable features as a model for human Pca, including initial androgen dependence, development from precursor (in situ) lesions, unequivocal metastases, and possible progressive neuroendocrine differentiation with progression (similar to possible increased neuroendocrine differentiation in advanced, potentially androgen insensitive tumors in humans) (48). Future studies on AA metabolism in the Tag mouse will likely include time course studies in this very promising 12T-10 line.

In addition, in collaboration with the laboratories of Dr. Matusik, Dr. Robert Coffey in Cell Biology, and Dr. Harold Moses, Director of the Vanderbilt-Ingram Cancer Center, we have been involved in the characterization of potential new mouse models of prostate carcinoma. These include mice with metallothionein promoter driven overexpression of rat TGFα (MT-TGFα) (49, 50) and a dominant negative truncated form of the TGFβ-receptor type II (MT-DNIIR) (51, 52). These mice show high levels of transgene expression in the prostate and develop lesions which look very similar to human high grade PIN, and in the case of some MT-DNIIR lines, foci suspicious for invasion (52). The generation and characterization of these mice and related transgenic constructs using the long probasin promoter have been the focus of recent grant applications, including a now funded NIH Mouse Consortium Grant (Robert Coffey, P.I., Scott Shappell, Co-I, 10 % effort) and a submitted Dept. of Defense Prostate Cancer Center Grant (Robert Matusik, P.I., Scott Shappell, P.I. of Path Core Lab, 25 % effort). Characterization of AA metabolism in these mice, supported in part by observations in the Tag mouse, are beyond the scope of the current grant and separate funding has already been sought for this endeavor in my recent application for a Culpeper Foundation Medical Scholarship.

B) AA metabolism assays on prostate tissue from specific mouse lines at time points indicated above, using HPLC as in human prostate studies (Task 1, part A) (months 3-12).

Research accomplishments: Corroborating our lack of detection of 8-LOX immunostaining in the prostate of the Tag mouse, 8-LOX mRNA by RT-PCR (not shown) and 8-HETE formation has not been detected in AA incubations of wild type or 12T-7f Tag mouse prostates from 13 to 19 weeks (not shown). Similar to this approach in analysis of human prostate tissues, these assays allow for possible detection of other AA metabolites. In short HPLC runs, polar metabolites possibly representing prostaglandins (PGs) have not co-eluted with PG standards (not shown). COX-2 message has not been detected by RT-PCR in wild type mice or in most of the Tag mice 12T-7f examined thus far, although COX-2 mRNA was detected by RT-PCR in one of two 18 week and one of two 19 week 12T-7f mice (not shown). The most prominent alteration in AA metabolism identified thus far is in 12-HETE formation. Wild type prostate sythesizes 12-HETE from exogenous AA, particularly the anterior prostate. 12-HETE formation is much less pronounced in the dorsolateral lobe (most analagous to human peripheral zone) in wild type mice (1.7 % conversion from exogenous AA) (Figure 5a, Appendix 1). 12-LOX message is detected by RT-PCR at all time points examined thus far (13 - 19 weeks) in the Tag 12T-7f mouse dorsolateral prostate (not shown), and by enzyme assay, there is increased formation (approximately 2- to 7-fold) of 12-HETE from AA in the dorsolateral prostate of Tag 12T-7f mice compared to wild type (Figure 6b, Appendix 1).

These intriguing results remain to be further characterized, including immunohistochemical localization of the 12-LOX in the Tag mouse prostate. Future avenues for investigation include possible mechanisms whereby 12-HETE can contribute to tumor formation in the mouse, including via activation of murine PPAR species. An alternate hypothesis includes possible contribution to tumor angiogenesis, as this mechanism has been postulated for 12-HETE in growth of human prostate cancer cells injected into nude mice, supported by *in vitro* observations (4). The very cellular stroma of the 12T-7f Tag mouse line (33) shows immunophenotypic characteristics compatible with early vascular differentiation and administration of antibodies to vascular endothelial growth factor (anti-VEGF) to these animals results in reduced prostate volume (Dr. Tom Daniel, personal communication). This mouse line may represent a very powerful model for investigating the contribution of AA metabolism to tumor angiogenesis. Whether other alterations in AA metabolism are seen in the unique 12T-10 mouse remains to be determined.

Key Research Accomplishments

- Use of northern blots, western blots, and tissue incubations with HPLC to demonstrate the uniform presence in benign prostate of 15-LOX-2 mRNA, protein, and enzyme activity.

- Use of paraffin immunohistochemistry to characterize the distribution of 15-LOX-2 in normal prostate. 15-LOX-2 is expressed in apical or secretory cells, particularly in the peripheral zone.

- Use of immunohistochemistry and enzyme assays to demonstrate a marked reduction or loss of 15-LOX-2 expression in prostate adenocarcinoma compared to benign prostate.

- Utilization of snap frozen prostate tissues to extend studies to RNA analysis. Demonstration by northern blots that 15-LOX-2 mRNA is reduced in prostate cancer compared to benign in the vast majority of cases.

- Use of immunohistochemistry and radical prostatectomy whole mount paraffin blocks to characterize possible loss of 15-LOX-2 in PIN and to correlate loss of 15-LOX-2 with tumor grade (data pending).

- Use of enzyme assays (tissue incubation and RP-HPLC) to demonstrate that significant 5-HETE or 12-HETE production is not observed in benign or malignant prostate tissues.

- Use of Rnase protection assays to detect COX-2 in a subset of prostate adenocarcinomas.

- Use of RT-PCR to demonstrate constituitive expression of PPARα, PPARβ, and PPARγ in benign prostate and prostate adenocarcinoma.

- Completion of initial experiments demonstrating that 15-HETE induces PPARγ dependent transcription in prostate carcinoma cell lines.

- Demonstration by immunohistochemistry, RT-PCR, and enzyme assays that the 8-LOX murine homologue of 15-LOX-2 is not expressed in wild type or the 12T-7f line of the Tag transgenic mouse model of prostate adenocarcinoma.

- Demonstration by enzyme assay that 12-HETE formation is the most characteristic aspect of AA metabolism in the 12T-7f Tag mouse.

- Establishment of a snap frozen tissue bank of prostate from radical prostatectomies performed at Vanderbilt University Medical Center.

Reportable Outcomes

Manuscripts:

Shappell, S.B., Boeglin, W.E., Olson, S.J., Kasper, S., Brash, A.R.: 15-Lipoxygenase-2 (15-LOX-2) is expressed in benign prostatic epithelium and reduced in prostate adenocarcinoma. Am. J. Pathol., 155:235-245, 1999.

Carter, B.A., Brash, A.R., Page, D.L., Olson, S.J., **Shappell, S.B.**: 15-lipoxygenase-2 expression in benign, precursor, and invasive cancer breast lesions. Correlation with apocrine differentiation. In preparation.

Shappell, S.B., Keeney, D.S., Page, R., Boeglin, W.E., Olson, S.J., Brash, A.R.: Expression of 15-lipoxygenase-2 (15-LOX-2) in benign and neoplastic sebaceous glands. In preparation.

Abstracts:

Shappell, S.B., Boeglin, W.E., Olson, S.J., Brash, A.R. 15-lipoxygenase-2 (15-LOX-2) is expressed in benign prostatic epithelium and reduced in prostate adenocarcinoma. Lab. Invest. 79:107a, 1999.

Brash, A.R., Boeglin, W.E., Schoenhard, J.A., Keeney, D.S., Carter, B., **Shappell, S.B**. 15-lipoxygenase-2 in human epithelial cells and its reduced expression in cancer. Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation, and Related Diseases, 6th International Conference, Boston, MA, 1999.

Shappell, S.B., Manning, S., Boeglin, W.E., Jack, G.S., Guan, Y., Breyer, M.D., Wheeler, T.M., Scardino, P.T., Brash, A.R. Arachidonic acid metabolism in prostate adenocarcinoma. American Urological Association, 95th Annual Meeting, Atlanta, GA, 2000, to be submitted.

Presentations:

Shappell, S.B., Boeglin, W.E., Olson, S.J., Brash, A.R. 15-lipoxygenase-2 (15-LOX-2) is expressed in benign prostatic epithelium and reduced in prostate adenocarcinoma. Poster Presentation, 1999 Annual Meeting of the United States and Canadian Academy of Pathologists, San Francisco, CA. (March 1999)

Shappell, S.B. Arachidonic Acid Metabolism in Prostate Adenocarcinoma. Clinical Pharmacology Seminar Series, Vanderbilt University Medical Center, March, 1999.

Shappell, S.B. Arachidonic Acid Metabolism in Human and Murine Models of Prostate Adenocarcinoma. Investigative Pathology Research in Progress Seminar Series, Vanderbilt University Medical Center, April, 1999.

Brash, A.R., Boeglin, W.E., Schoenhard, J.A., Keeney, D.S., Carter, B., **Shappell, S.B**. 15-lipoxygenase-2 in human epithelial cells and its reduced expression in cancer. Platform Presentation, Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation, and Related Diseases, 6th International Conference, Boston, MA, 1999.

Tissue repository:

Tissue bank of snap frozen prostate for molecular biology studies, Vanderbilt-Ingram Cancer Center, Human Tissue Acquisition and Shared Resources.

Based on our success with the snap frozen cores obtained from Baylor College of Medicine, which have been used to generate the data described in this report, we have instituted a more aggressive program of obtaining tissue adequate for molecular biologic studies from radical prostatectomy cases performed at our own institution. Beginning in July, 1999, Greg Jack, a Vanderbilt medical student predoctoral fellow in my lab, and Kimberly Newsome-Johnson of the VICC have been procurring cores of prostate tissue in

the operating room, using a protocol similar to that reported previously by our colleagues at Baylor (35). 6 mm core biopsies are taken in each case from:

TZ, mid to apex, right
TZ, mid to apex, left
PZ, mid to apex, right
PZ, mid to apex, left
TZ, mid to base, right
TZ, mid to base, left
PZ, mid to base, right
PZ, mid to base, left
Seminal vesicle

Additional sections are occasionally taken from large grossly evident tumors. The defects left by the biopsy tool are identified by color coded ink to allow definitive identification in whole mount sections. The cores are identified as to benign, tumor, and percent tumor based on correlation with histopathologic findings in whole mount sections and this information is entered into a data base, along with pertinent clinical and pathologic features of the case. Thus far, over 300 samples from more than 30 radical prostatectomies have been successfully procurred. We expect this jointly created tissue repository to be a tremendous resource for prostate researchers.

Database:

Radical prostatectomy pathology data base. This includes clinical and all pathological parameters from all radical prostatectomy cases submitted in their entirety for whole mount sections and signed out by Dr. Shappell. This was begun Jan. 1998 and currently contains over 200 cases. Histopathologic parameters recorded include tumor location, grade, stage (including focal or established extracapsular extension, seminal vesicle status, and pelvic lymph node status), status of margins and location of positive margins, total tumor volume determined by digitized planimetry, and other unique histologic findings. This data base has greatly facilitated our own research efforts as well as expanded clinicopathologic research opportunities for members of the Dept. of Urology (e.g., see abstract, Appendix 4).

Funding applied for based on work supported by award:

Awarded:

Discovery Grant in Prostate Cancer Research; The Vanderbilt Cancer Center; **Principal Investigator:** Scott B. Shappell, M.D., Ph.D.; 04/01/99 - 03/31/00, $23,500.00 (total).

15-LOX-2 and PPARs in regulation of prostate carcinoma cell growth and differentiation; Vanderbilt University Medical Center Intramural Interdisciplinary Discovery Grant; **Principal Investigator:** Scott B. Shappell, M.D., Ph.D.; 07/01/99 - 06/30/01, $110,455.00 (total); 5 % salary support.

Submitted, not awarded:

Modulation of 15-lipoxygenase-2 by hormonal therapy and the role of lipoxygenases and cyclooxygenases in prostate adenocarcinoma: A combined immunohistochemical and laser capture microdissection RT-PCR analysis; NIH Finasteride Chemoprevention Trial / Southwest Oncology Group; **Principal Investigator:** Scott B. Shappell, M.D., Ph.D; 07/01/99 - 06/30/0, $165,958.00 (total); 10 % effort

(No grants were awarded in response to this RFA; an additional RFA will be released pending completion of the PCPT trial, for which we anticipate a modified re-submission)

Submitted, pending:

Arachidonic acid metabolism and peroxisome proliferator activated receptors in prostate carcinoma; Charles E. Culpeper Foundation Scholarship in Mecical Science; **Principal Investigator**: Scott B. Shappell, M.D., Ph.D.; 07/01/00 - 06/30/03; 99,925.00/yr (direct); 25 % effort.

Conclusions

The funding established by this New Investigator Award has contributed to the establishment of a new laboratory, provided salary support for an experienced research technician, and allowed us to make tremendous progress in investigating the originally proposed hypotheses. We have made substantial progress in each of the proposed areas of investigation and are ahead of schedule in some areas. We have obtained substantial data which has clearly supported our hypothesis that 15-LOX-2 is expressed in benign prostate and reduced or lost in prostate carcinoma. Given the possible role of 15-HETE in regulating cell function, its reduced expression in prostate is perhaps a crucial specific molecular alteration contributing to the malignant phenotype. Prostate cancer is clearly a genetically and molecularly heterogeneous disease. Our results have indicated that while loss of 15-LOX-2 may be the major alteration in AA metabolism in Pca, it is not universally observed or pronounced in all cases. Our experimental approaches will allow for detection of altered expression of other AA metabolizing enzymes in a potentially minor fraction of tumors. It is likely that these changes contribute to regulation of cell proliferation, altered cellular differentiation, tumor cell invasion, or modification of the tumor stroma and angiogenesis, and that these changes may be differentially important in different stages of Pca development or progression. Understanding how these novel signaling mechanisms interact with other aspects of prostate regulation will ultimately allow for therapeutic avenues tailored to individual tumor characteristics.

By immunohistochemistry, enzyme assays using reverse phase HPLC, western blots, and northern blots we have demonstrated that 15-LOX-2 is uniformly present in benign prostate. It is expressed in apical secretory cells primarily of the peripheral zone and to a lesser degree, also in the transition zone and central zone. Such cells are the differentiated counterpart of the malignant acinar cells in Pca. Using these techniques, applied to snap frozen prostate tissues with optimal RNA preservation and rigidly assessed as to their benign and malignant nature, we have shown that 15-LOX-2 mRNA and enzyme activity are reduced in the majority of Pca cases. Ongoing studies are examining correlation of this phenomenon with grade, stage, and other prognostic factors. Regardless of correlation with pathologic parameters or outcome, we hypothesize that this alteration is etiologically crucial in a high percentage of tumors.

Combined with results from other laboratories showing possible 15-HETE activation of PPARγ in in vitro transcription assays and inhibition of proliferation of Pca cell lines by synthetic PPARγ agonists, our data has thus far supported our original hypothesis that 15-HETE may be an endogenous ligand for this class of nuclear receptors in the prostate. By sensitive RT-PCR assays, we have shown that benign and malignant prostate uniformly constituitively expresses all three PPAR subtypes (α, β, and γ). 15-HETE appears to be able to activate PPARγ-dependent transcription in Pca cell lines and ongoing experiments are addressing the effect of this signaling pathway on Pca cell proliferation, cell cycle, and differentiation. In related studies not funded directly by this proposal, we have found that 15-LOX-2 is uniformly expressed in benign sebaceous glands, cells regulated by androgens and which appear to also express PPARγ. We have also found expression of 15-LOX-2 in a subset of breast lesions showing apocrine differentiation, which also possess androgen receptors, and PPARγ-agonists induce differentiation of breast cancer cell lines. The combined results suggest very novel interactions between androgens, AA metabolism, and PPAR nuclear receptors, which should provide insight into regulation of prostate cancer cell differentiation, both AR responsive and advanced AR insensitive.

We have also generated data directly related to the postulated presence of other AA metabolizing enzymes in some Pca. Our results with 5-LOX and COX-2 should indicate whether results generated by others in cell lines are actually applicable to real prostate tumors. Our 12-HETE analyzes provide information supplementing previous reports on 12-LOX message in Pca and cell lines. Increased 12-HETE formation is the major alteration in AA metabolism detected thus far in a transgenic mouse model of Pca. Understanding how these enzymes are modified in 15-LOX-2 positive or negative tumors should contribute to our overall understanding of the spectrum of changes in AA metabolism in Pca.

We have investigated potential alterations in the murine homoloque of 15-LOX-2, an 8-LOX, in the long probasin promoter-SV40 (Tag) transgenic mouse model of Pca. The originally proposed lines uniformly developed in situ lesions, but only one line inconsistently developed invasive disease. We have not observed 8-LOX in wild type or this 12T-7f line. However, newly characterized lines (e.g., 12T-10) are more suitable analogs of human Pca, developing high grade precursor lesions, invasive disease, and consistent metastatic lesions. In addition to completing studies in the 12T-7f mouse, we anticipate doing a complete study on AA metabolism in the 12T-10 mouse with the funds from this grant application. The potential role of increased 12-HETE detected thus far in the Tag mouse model remains to be elucidated, including whether this functions as a ligand for PPAR in the mouse or contributes to tumor development and progression through other mechanisms. Speculated contribution to tumor angiogenesis, based on results in human Pca cell lines, is an intriguing hypothesis given the stromal alterations in this line. Clearly this mouse model and its different sublines provide a tremendously unique and potentially powerful approach for further clarifying the contribution of these lipid derived mediators in Pca tumor biology.

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APPENDIX I — FIGURES

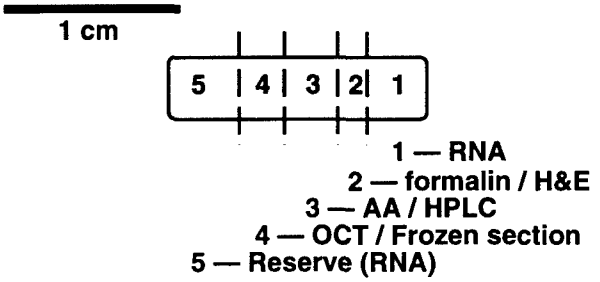

Figure 1: Processing of snap frozen 6 mm punch biopsies of prostate.

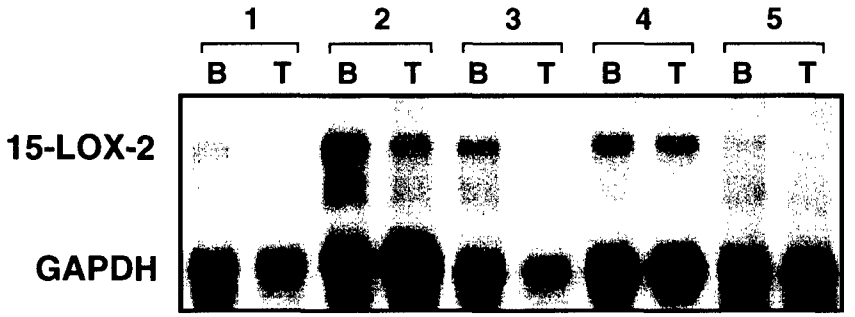

Figure 2: Northern blot of 15-LOX-2 and housekeeping gene GAPDH in benign (B) and tumor (T) snap frozen prostate tissue from five different patients. 20 ug total RNA, probed with a 1069 bp 15-LOX-2 cDNA. 15-LOX-2 mRNA is visibly reduced in tumor compared to benign in most cases, and by normalization to GAPDH (determined by densitometry on film or phosphorimager) in 10 of 11 cases examined thus far, including case in lane 4. Tumors in illustrated cases were: 1) Gleason 9, 2) Gleason 8, 3) Gleason 5, 4) Gleason 6, and 5) Gleason 8.

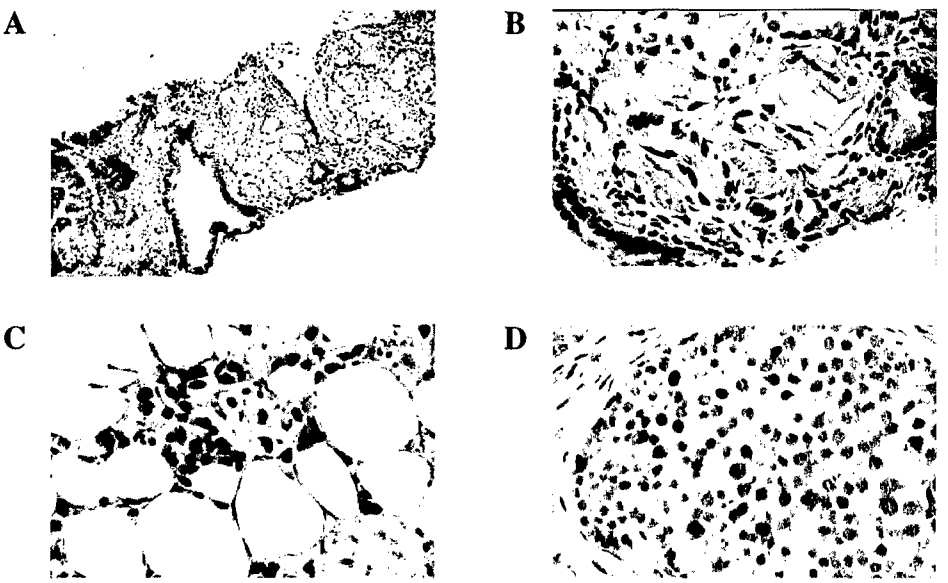

Figure 3: **(A)** 15-LOX-2 immunostaining on standard formalin fixed paraffin embedded 18-gauge prostate core biopsy. Strong immunostaining of benign glands (left and bottom center) and complete absence of immunostaining in small focus of Gleason grade 3 + 3 = 6 adenocarcinoma (center and right) (original magnification, 100x). **(B)** Higher magnification, with absent tumor staining (center) vs. positive staining for 15-LOX-2 in adjacent benign glands (right and left) (original magnification, 400x). Paraffin immunoperoxidase using 1:2500 dilution of rabbit anti-human 15-LOX-2. **(C)** Paraffin immunoperoxidase PPARγ immunostaining in invasive breast cancer (positive control). **(D)** PPARγ immunostaining in 5-10% of nuclei in cribriform Gleason pattern 4 prostate cancer. Primary antibody, clone E8 (Santa Cruz Biotech., Santa Cruz, CA), 1:10. (Original magnification 400x).

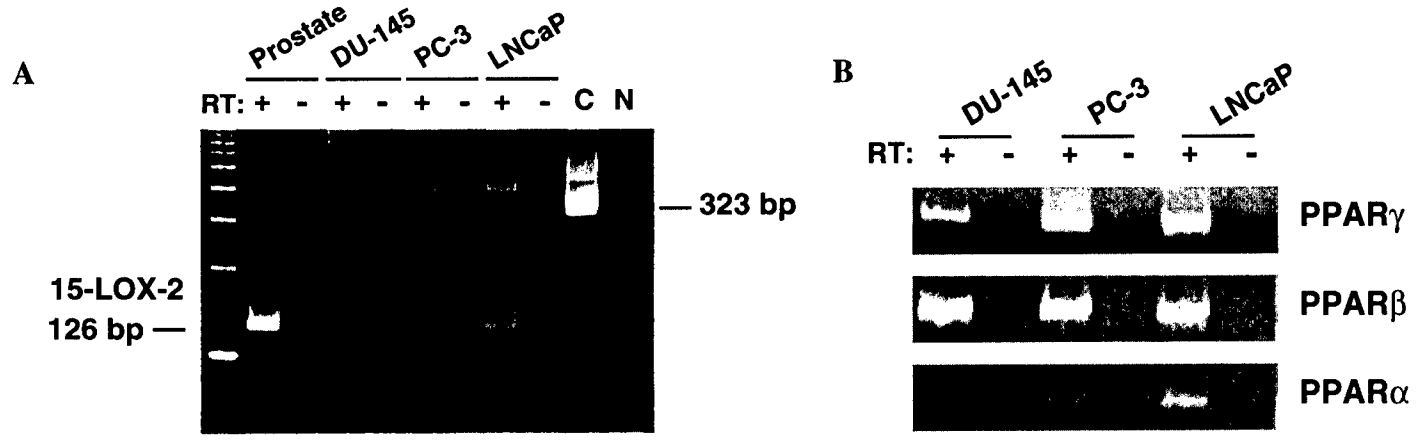

Figure 4: **(A)** 15-LOX-2 mRNA expression in benign prostate vs. prostate carcinoma cell lines, DU-145, PC-3, and LNCaP. RT-PCR beginning with 5 ug total RNA. The expected 126 bp fragment for 15-LOX-2 is indicated, with strong signal in benign prostate vs. lack of expression in PC-3 and weak expression in DU-145 and LNCaP. RT-PCR using Promega Access RT-PCR System, with 30 cycles. Paired + and - lanes indicate presence or absence of reverse transcriptase, controlling for any contaminating genomic DNA. Products resolved on 5 % polyacrylamide gels. C indicates positive control provided by manufacturer. N indicates negative control, with no template. **(B)** PPAR mRNAexpression in prostate carcinoma cell lines. RT-PCR beginning with 5 μg total RNA. +, - indicate presence or absence of reverse transcriptase (primers provided by Dr. Ray DuBois).

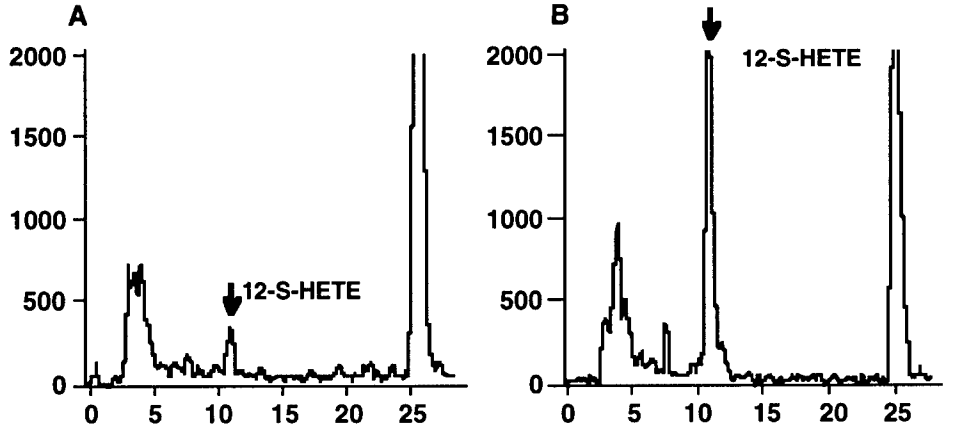

Figure 5: AA metabolism in dorsolateral lobes of wild type **(A)** and LPB-Tag mouse **(B)**, showing increased 12-HETE formation in incubation of tumor from Tag mouse compared to wild type prostate.

Appendix 2

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15-Lipoxygenase-2 (15-LOX-2) Is Expressed in Benign Prostatic Epithelium and Reduced in Prostate Adenocarcinoma

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Human 15S-lipoxygenase-2 (15-LOX-2) is a recently identified lipoxygenase that has approximately 40% sequence identity to the known human 5S-, 12S-, and 15S-lipoxygenases. 15-LOX-2 has a limited tissue distribution, with mRNA detected in prostate, lung, skin, and cornea, but not in numerous other tissues, including peripheral blood leukocytes. In the current study, we have characterized the distribution of 15-LOX-2 in the human prostate by immunohistochemistry, demonstrated the ability of benign prostate tissue to form 15S-hydroxyeicosatetraenoic acid (15S-HETE) from exogenous arachidonic acid (AA), and begun characterizing possible alterations in 15-LOX-2 in prostate adenocarcinoma. Incubation of benign prostate tissue with [^{14}C]AA resulted in formation of [^{14}C]15-HETE, as determined by reverse- and straight-phase high-performance liquid chromatography. 15-HETE was the major AA metabolite formed. By immunohistochemistry, 15-LOX-2 is located in secretory cells of peripheral zone glands and large prostatic ducts and somewhat less uniformly in apical cells of transition and central zone glands. 15-LOX-2 was not detected in the basal cell layer, stroma, ejaculatory ducts, seminal vesicles, or transitional epithelium. Immunostaining of 18 radical prostatectomy specimens showed a loss of 15-LOX-2 in the majority of prostate adenocarcinomas; 14 of 18 cases showed loss of 15-LOX-2 in >25% of the tumor (mean, 74.9% negative for 15-LOX-2; range, 38.9% to 100%). Incubation of paired pure benign and pure malignant prostate tissue from the same radical prostatectomies showed that 15-HETE formation was markedly reduced (>90%) or undetectable in incubations of prostate adenocarcinoma. 15-LOX-2 is a novel human lipoxygenase with a limited tissue distribution that is strongly expressed in benign prostate glandular epithelium and lost to a variable degree in the majority of prostate adenocarcinomas. *(Am J Pathol 1999, 155:235–245)*

Arachidonic acid (AA) metabolites are important mediators of a variety of physiological processes and inflammatory reactions. In addition, alterations in AA metabolism may potentially mediate key steps in certain neoplastic processes.[1–3] AA is metabolized via cyclooxygenase to prostaglandins, prostacyclin, and thromboxane,[4] and via lipoxygenases (LOX) to hydroxyeicosatetraenoic acids (HETEs) or leukotrienes (5-LOX pathway).[5,6] Until recently, three lipoxygenases were recognized in humans: a 5S-LOX found in leukocytes, a 12S-LOX found in platelets and certain epithelia, and a 15S-LOX in reticulocytes, eosinophils, macrophages, and skin.[7] Recently, in studying lipoxygenase expression in human skin, Brash et al[8] discovered a second 15S-lipoxygenase (herein referred to as 15-LOX-2). The cDNA-derived amino acid sequence of 15-LOX-2 showed only 44% identity to 5-LOX and 38% to 39% identity to 12-LOX and the reticulocyte type of 15-LOX (herein referred to as 15-LOX-1).

In addition to structural differences, 15-LOX-2 exhibits differences in certain enzymatic characteristics. In contrast to the reticulocyte type of 15S-lipoxygenase (15-LOX-1), 15-LOX-2 converts AA exclusively to 15S-hydroperoxyeicosatetraenoic acid (15-HPETE); this hydroperoxide is reduced by cellular peroxidases to 15S-hydroxyeicosatetraenoic acid (15-HETE). Also unlike 15-LOX-1, this newly characterized enzyme metabolizes linoleic acid poorly compared with AA.[8] The 15-LOX-2 cDNA was cloned from human hair roots.[8] In addition, 15-LOX-2 mRNA was detected in a limited number of tissues, including prostate, lung, and cornea. In contrast to the distribution of 15-LOX-1, 15-LOX-2 was not detected in peripheral blood leukocytes, nor was it detected in liver, kidney, spleen, thymus, testis, ovary, skeletal muscle, heart, brain, or intestinal tissue.[8]

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The limited tissue distribution of 15-LOX-2 suggests a possible role in regulation of organ-specific functions or differentiation or possible alterations in disease states. In continuing efforts to elucidate potentially important molecular alterations in prostate cancer development or progression, a limited number of recent investigations using both human tissues and prostate carcinoma cell lines have begun focusing on AA-metabolizing enzymes, including cyclooxygenase,[9] 12-LOX,[10] 5-LOX,[11] and 15-LOX-1.[12] In the current study, we have characterized the expression of 15-LOX-2 in benign prostate by immunohistochemistry, demonstrated the ability of benign prostate to form the enzymatic product of 15-LOX-2 (15-HETE) from AA, and begun exploring the possible altered expression of 15-LOX-2 and 15-HETE formation in prostate adenocarcinoma.

Materials and Methods

Case Selection and Histology

Fresh tissue was procured from radical prostatectomy or cystoprostatectomy specimens accessioned in the Surgical Pathology Laboratory at the Vanderbilt University Medical Center. For prostatectomies, the external aspects (surgical margins) were inked according to standard protocols and whole cross sections (perpendicular to the apex-base axis) were made. Based on gross examination, possible benign and tumor regions were separately excised in such a manner as to not compromise surgical pathological evaluation of the specimen. Fresh tissues were placed in liquid nitrogen or immediately placed in a −80°C freezer. Before utilization in enzyme assays, thin sections parallel to the longest axis of the tissue were removed, and frozen or routine formalin-fixed paraffin-embedded H&E-stained sections were prepared. The samples were assessed as to percentage of benign glandular, stomal, and adenocarcinoma tissue present. Although numerous samples of benign tissue were thus generated, owing to the known difficulty of recognizing prostate carcinoma grossly, only a subset of presumably malignant tissues so procured turned out to be 100% tumor. For enzyme assays, frozen normal tissue samples and pairs of pure benign glands and pure carcinoma admixed with stroma were used according to availability.

Whole-mount sections were processed for histology and subjected to standard surgical pathology evaluation. Cases that were used for AA incubations were also used for 15-LOX-2 immunohistochemistry. Additional random cases were selected to give a representative mixture of tumor grades with various combinations of organ-confined disease and extra-capsular extension (ECE) with or without seminal vesicle (SV) and margin involvement: five cases of Gleason grade 5 or 6 without ECE or SV involvement, three cases of Gleason grade 6 with ECE or SV involvement, four cases of Gleason grade 7 without ECE or SV involvement, three cases of Gleason grade 7 with ECE or SV involvement, and three cases of Gleason grade 8 to 10 with ECE or SV involvement. Numerous foci of well differentiated carcinoma (Gleason patterns 1 and 2) were also included (generally incidental in transition zone).

Preparation of 15-LOX-2 for Antibody Production

cDNA encoding the open reading frame of 15-LOX-2 was cloned into the pET3a vector and expressed in phage-induced *Escherichia coli* (HMS174 cells). Ten milliliters of overnight culture of HMS174 cells transformed with pET3a/15-LOX-2 in LB medium containing 50 μg/ml ampicillin was used to inoculate a fresh 500-ml culture of LB/ampicillin. Incubation was continued at 37°C until the absorbance at 600 nm reached 0.9 A. One hundred milliliters of phage culture (CE6, expressing T7 RNA polymerase) was then added together with 5 ml of 1 mol/L $MgSO_4$, and incubation was continued for 3 hours at 37°C. Under these incubation conditions, overexpression of the 15-LOX-2 protein gave a catalytically inactive lipoxygenase, comprising 25% to 50% of the cellular protein. The bacterial pellets were collected by centrifugation, washed with phosphate-buffered saline (PBS), and resuspended in 40 ml of PBS, and the cells were disrupted by sonication. The insoluble fraction containing the majority of the lipoxygenase protein was collected by centrifugation and washed once with 1.5 mol/L NaCl followed by a wash with 1% Triton X-100 in PBS. The pellet was again washed in PBS, dissolved in 8 ml of loading buffer, and subjected to semipreparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% polyacrylamide gels with 4% stacking gels. The clearly visible band of 15-LOX-2 protein was cut from Coomassie-blue-stained gels, placed in a dialysis bag, and electroeluted. The protein was lyophilized, and an aliquot was quantified by its appearance on SDS-PAGE using bovine serum albumin as standard.

Preparation and Characterization of Polyclonal Antibody to 15-LOX-2

Rabbits were injected at six sites of back skin using an initial 0.1 mg of 15-LOX-2 per animal in 1:1 PBS/Freund's complete adjuvant. A booster injection of 0.1 mg using PBS/Freund's incomplete adjuvant was given 19 days later, and blood serum was collected beginning 14 days later. High-titer bleeds from a single rabbit were used for the Western analyses and immunohistochemistry reported herein.

The specificity of the rabbit antibody for 15-LOX-2 and possible cross-reactivity to other human lipoxygenases was examined by Western blotting using five human lipoxygenases. 15-LOX-1, 15-LOX-2, and 12R-LOX[13] were prepared by bacterial expression, each including an amino-terminal histidine tag. The proteins were recovered using a nickel affinity column (Qiagen, Chatsworth, CA) essentially according to the manufacturer's instructions. Purified human 5-lipoxygenase was a generous gift from Dr. Denis Reindeau (Merck-Frosst). Aliquots of these four

lipoxygenase proteins were run on SDS-PAGE, and after staining with Ponceau S, the samples were quantified by visual inspection relative to dilutions of a standard of bovine serum albumin. Aliquots of 50 or 5 ng were used for Western analysis. A partially purified preparation of human platelet 12-lipoxygenase from Oxford Biomedical Research (Oxford, MI) was used as a source of human 12*S*-LOX (platelet 12-LOX). For Western blots employing 5 ng of purified lipoxygenases, 8 μg of protein of the partially purified 12*S*-LOX was used, which, assuming the 12-LOX comprises at least 0.1% of the extract, corresponds to at least 8 ng of 12*S*-LOX protein. Adequate protein loading of this sample was further confirmed by staining of gels and visual inspection. Proteins were separated by 12% SDS-PAGE electrophoresis and transferred to nitrocellulose membranes using standard conditions.

The Western analyses were carried out using 15-LOX-2 polyclonal antibody (1:6000) and an alkaline-phosphatase-conjugated affinity-purified donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA; 1:7500 in bovine serum albumin/Tris-buffered saline/0.1% Tween-20) and Tris-buffered levamisole (Sigma Chemical Co., St. Louis, MO) followed by nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate for development.[14]

Immunohistochemistry and Quantitation of 15-LOX-2 Immunostaining

Whole-mount sections that contained adequate benign tissue and tumor typical of the overall specimen were selected. In general, both right and left halves of the respective whole-mount sections were recut onto separate immuno-slides for subsequent immunohistochemical staining. In some cases, multiple whole-mount blocks were used for immunostaining. Paraffin immunoperoxidase studies were performed using a 1:2500 dilution of either primary rabbit antisera for 15-LOX-2 or preimmune sera on an automated immunostainer (Ventana 320 automated immunohistochemistry system, Ventana Medical Systems, Tucson, AZ) with an avidin-biotin complex method without antigen retrieval techniques.[15] 15-LOX-2 immunostaining in benign prostate tissue was uniform and is expressed descriptively or semiquantitatively as 0 to 4+: 0, no staining; 1+, focal weak staining; 2+, weak to moderate intensity staining in up to one-half of the cells in most glands or most cells in up to one-half of the glands; 3+, moderate to intense staining in greater than one-half of the cells or one-half of the glands but less than strong, uniform staining; 4+, strong uniform staining in essentially all cells. 15-LOX-2 immunostaining was quantitated in tumor portions by carefully mapping out with ink on the glass slides the areas of tumor that were positive and negative and then by making enlarged photocopies of such outlines. Areas were determined using a digitized graphics tablet (WACOM) and NIH Image Analysis software. Results are expressed as the percentage of tumor negative for 15-LOX-2 immunostaining.

Tissue Incubations and HPLC Analysis

A 50- to 100-mg amount of benign or tumorous prostate tissue was homogenized in 4 vol of buffer (50 mmol/L Tris with 100 mmol/L NaCl and 100 μmol/L $CaCl_2$, pH 7.4). Incubations were begun by addition of 50 μmol/L [1-^{14}C]arachidonic acid (final concentration; New England Nuclear, Boston, MA) in $\leq$1% (final v/v) ethanol. Samples were incubated for 1 hour at 37°C with continual agitation and terminated by addition of 2.5 vol of cold methanol. After addition of 1.25 vol of dichloromethane, the sample was centrifuged to remove the protein precipitate, and the products were recovered in the mixed phase of methanol/water/dichloromethane. The sample was evaporated under a stream of nitrogen to remove most of the dichloromethane and methanol, water was added, and the products were recovered by C18 Sep-Pak extraction.[8] The extracts were analyzed by reversed-phase HPLC using a Beckman Ultrasphere 5-μm ODS column (25 × 0.46 cm) with a solvent of methanol/water/glacial acetic acid, either 90:10:0.01 (by volume) at a flow rate of 1.1 ml/minute (retention time of 15-HETE was approximately 5 minutes) or 80:20:0.01 at 1.1 ml/minute (retention time of 15-HETE approximately 15 minutes). Unlabeled HETEs (5-, 8-, 9-, 11-, 12-, and 15-HETEs) were added to each sample before HPLC analysis; this permitted an exact determination of the retention times of each HETE product within each individual chromatographic run. Ultraviolet (UV) spectra and the profiles at 205, 220, 235, and 270 nm were recorded using a Hewlett-Packard 1040A diode array detector, and radioactivity was monitored on-line using a Radiomatic Instruments Flo-One detector. Product formation in some incubations of benign and malignant prostate tissue pairs was also analyzed in more detail using reverse-phase HPLC with a solvent system of methanol/water/glacial acetic acid (75:25:0.01, by volume) at a flow rate of 1.01 ml/minute (retention time of 15-HETE approximately 31.5 minutes), with collection of 0.5-minute fractions and 10-minute scintillation counting of all fractions. In some samples, the main radiolabeled peaks were further characterized by normal-phase HPLC using a Beckman Ultrasphere 5-μm silica column (25 × 0.46 cm) and a solvent of hexane/isopropyl alcohol/glacial acetic acid 100:1:0.1 (by volume) at a flow rate of 1.1 ml/minute with UV detection using the diode array detector and on-line radioactive detection.

Results

15-HETE Formation by Benign Prostate Tissue

Incubations of benign prostate tissue homogenates with [^{14}C]AA resulted in the formation of 15-HETE in all cases examined (n = 8; Figure 1). Identity of this product was demonstrated by its precise co-elution on reverse-phase HPLC (and on straight-phase HPLC in selected samples) with co-injected unlabeled 15-HETE (see Materials and Methods). The addition of a mixture of unlabeled HETEs to each sample allowed an exact definition of the reten-

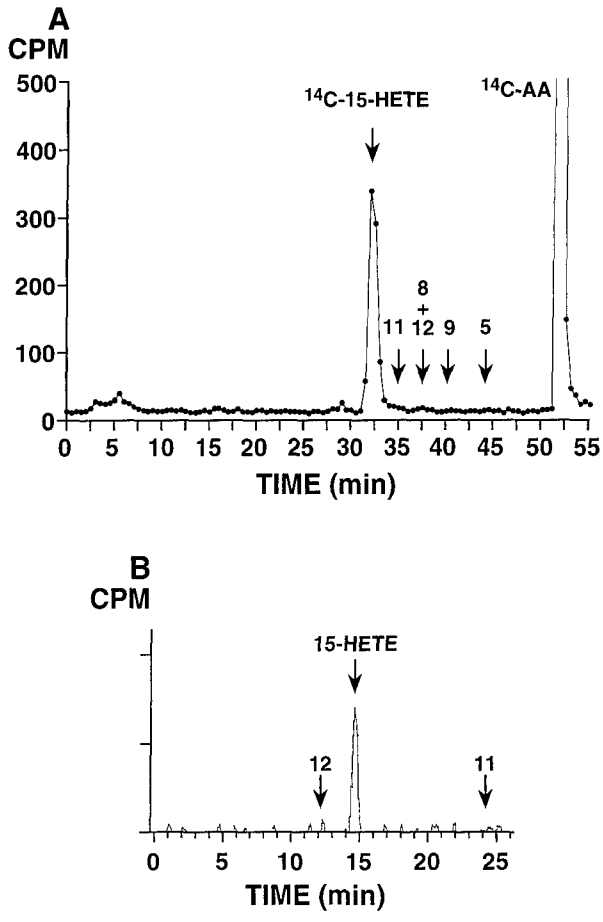

Figure 1. [^{14}C]AA metabolism in benign prostate tissue, showing prominent formation of [^{14}C]15-HETE. **A**: Reverse-phase HPLC analysis using a Beckman Ultrasphere 5-μm ODS column (25 × 0.46 cm) with a solvent of methanol/water/glacial acetic acid (75:25:0.01, by volume) at a flow rate of 1.01 ml/minute switched to 100% methanol at 40 minutes; 0.5-minute fractions were collected and subjected to scintillation counting. **Arrows** show retention times of unlabeled HETE standards co-injected with the ^{14}C sample. The experiment shown is representative of eight individual benign specimens analyzed. **B**: Normal-phase HPLC analysis of fractions 63 through 66 (31.5 to 33 minutes) from reverse-phase analysis in **A**, using a Beckman Ultrasphere 5-μm silica column (25 × 0.46 cm), a solvent system of hexane/isopropanol/glacial acetic acid (100:1:0.1, by volume) with a flow rate of 1.1 ml/minute and on-line detection of radiolabeled products using a Packard Flo-One Radiomatic detector. **Arrows** indicate retention times of co-injected unlabeled HETE standards.

tion times within each chromatographic run. The identity of the radiolabeled product as 15-HETE was further confirmed by the characteristic UV spectrum of the mixture of [^{14}C]15-HETE and the co-injected authentic standard[16]; the UV absorbance of the 15-HETE peak was distinctly elevated by the contribution from the [^{14}C]15-HETE. The amount of 15-HETE produced under these incubation conditions was variable. However, in all cases, 15-HETE was the major product detected, with variably sized peaks in the prostaglandin region (Figures 1 and 3). From analysis of these eight samples of benign prostate, minor amounts of other hydroxyeicosatetraenoates were occasionally detected; the most prominent of these additional HETE peaks, present in only one sample, was the one, tentatively identified as 12-HETE, eluting at 36 minutes in Figure 3B.

15-LOX-2 Immunostaining in Benign Prostate

The polyclonal antibody prepared against purified 15-LOX-2 was demonstrated to be specific for 15-LOX-2 and to not cross-react with other pertinent lipoxygenases by Western blotting. Whereas a strong immunoreactive band was obtained with purified 15-LOX-2 protein (5 ng), no immunoreactivity was seen with purified 15-LOX-1 or 5-LOX (5 or 50 ng) or a sample of partially purified 12*S*-LOX (not shown). Weak immunostaining was seen with a purified preparation of a recently identified 12*R*-LOX,[13] which may be expected, given the greater percentage of identity between 15-LOX-2 and 12*R*-LOX (approximately 50%) than between 15-LOX-2 and 5-LOX, 12*S*-LOX, and 15-LOX-1 (approximately 40%).[8,13] However, unlike 12*S*-LOX, which has been reported in prostate (see Discussion), 12*R*-LOX has not been identified in prostate. Hence, minimal cross-reactivity of a 15-LOX-2 antibody with a 12*R*-LOX is not an issue in prostate immunostaining, as benign prostate makes large amounts of 15-HETE (as reported herein), and prostate has not been observed to make 12*R*-HETE.

The cellular location of 15-LOX-2 was identified as ductal and acinar epithelium by immunohistochemistry. Benign prostatic epithelium consistently stained strongly and uniformly with the polyclonal antiserum against human 15-LOX-2 by paraffin immunoperoxidase (Figure 2A). Stromal, vascular, and inflammatory cells were uniformly negative (Figure 2B). The distribution of immunostaining was carefully examined in the benign portions of prostate sections from radical prostatectomies performed for prostate adenocarcinoma. There was strong uniform staining of apical (secretory) cells in peripheral zone glands, with essentially all cells staining in all glands (Figure 2, A and C). The pattern of staining was predominantly cytoplasmic granular, with frequent nuclear staining also evident (Figure 2C). Occasional detached cells in glandular and ductal lumens showed immunostaining, but corpora amylacea were negative. Basal cells did not stain (Figure 2C); this lack of staining was evident also in areas of basilar hyperplasia (not shown). Strong uniform 15-LOX-2 immunostaining was present in columnar secretory cells of large periurethral ducts and secondary prostatic ducts (Figure 2, D and E). In contrast, transitional epithelium of the prostatic urethra, periurethral ducts, and areas of transitional metaplasia was completely negative (Figure 2E).

Some variability in the intensity and extent of prostate glandular epithelial staining was noted in different anatomical zones and benign histological alterations. Although most glands in the transition zone showed moderately intense staining in the majority of cells, staining was appreciably less uniform and less intense than seen in peripheral zone glands (Figure 2F). This slightly reduced 15-LOX-2 immunostaining was noted in variably prominent nodules of transition zone glandular hyperplasia (the histological correlate of benign prostatic hyperplasia, or BPH), including in cystically dilated hyperplastic glands. In general, staining was stronger in glands in the periphery of such nodules, with lesser staining, including negative glands, in the center of hyperplastic

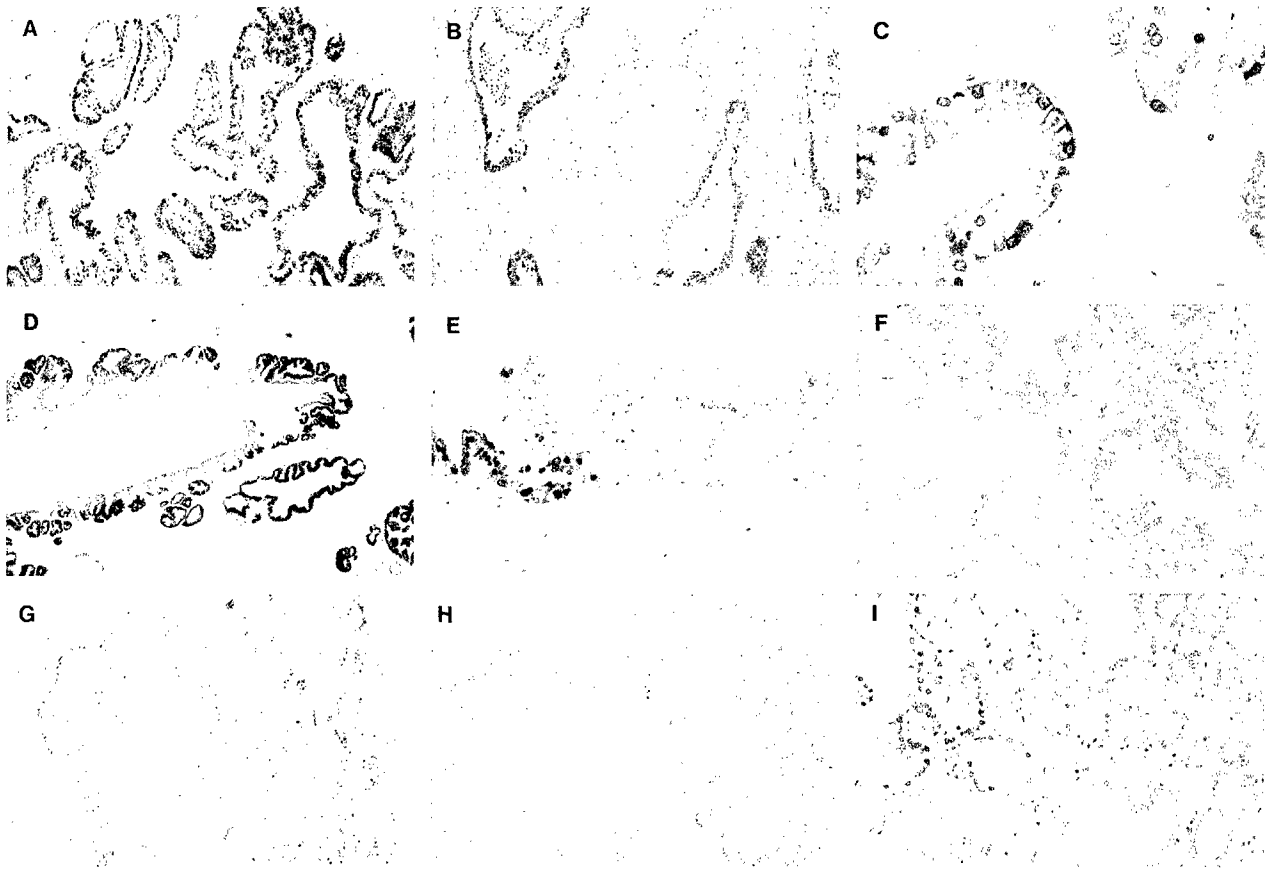

Figure 2. 15-LOX-2 immunostaining in benign prostate. **A**: Uniform strong staining of glandular epithelium (original magnification, ×200). **B**: Absent immunostaining in vein and artery (middle), fibromuscular stroma, and admixed chronic inflammatory cells. Strong staining of glands in right and left of field (×100). **C**: Higher magnification showing cytoplasmic granular and focal nuclear staining of apical (secretory) epithelial cells (×400). **D**: Staining of secretory cells in large prostatic duct (×100). **E**: Higher magnification showing positive staining in columnar secretory cells (left) and lack of staining in transitional epithelium (right) of prostatic duct (×200). **F**: Non-uniform and reduced intensity staining in hyperplastic glands in transition zone (×200). **G**: Focal staining of glands in central zone (region around ejaculatory ducts toward base of gland; ×100). **H**: Complete absence of staining of ejaculatory duct (lumen toward lower right; ×100). **I**: Absent staining in atrophic glands (right). Compare with positive immunostaining of more normal-appearing glands, with ample cytoplasm, in left of photomicrograph (×200). Paraffin immunoperoxidase staining using rabbit polyclonal anti-15-LOX-2, 1:2500.

nodules. Compared with the completely uniform strong staining in essentially all peripheral zone glands, benign glands in the central zone (surrounding the ejaculatory ducts) also showed slightly reduced intensity and frequency of 15-LOX-2 immunostaining (Figure 2G). Ejaculatory ducts and seminal vesicles were uniformly negative (Figure 2H). Reduced and even absent immunostaining was also noted in atrophic glands in the peripheral zone (Figure 2I), with intermediate staining intensity occasionally noted in partially atrophic glands. No immunostaining was detected in any glandular elements (or any other tissue compartment) with negative preimmune serum controls (n = 5; data not shown). In addition, staining of benign prostate glands was eliminated by preincubation of antisera with purified 15-LOX-2 protein[8] (not shown). A summary of the semiquantitative 15-LOX-2 immunohistochemical staining in benign prostate is shown in Table 1.

Decreased or Absent 15-LOX-2 Enzyme Activity in Prostate Adenocarcinoma

15-LOX-2 activity was assessed in benign prostate tissue and prostate adenocarcinoma frozen from the same rad-

Table 1. 15-LOX-2 Immunostaining in Benign Prostate

Prostate compartment	15-LOX-2 Immunostaining (0–4+)	Number of informative cases
Peripheral zone glands	4+	18
Atrophic glands	0–2+	9
Transition zone glands (BPH)	2+	7
Central zone glands	2–3+	4
Periurethral/prostatic ducts*	4+	7
Stroma and inflammatory cells	0	18
Transitional epithelium; ejaculatory ducts; seminal vesicles	0; 0; 0	6; 4; 5

*Columnar/secretory (not transitional) epithelial cells.

ical prostatectomy specimens. In all benign samples, incubation with [^{14}C]AA resulted in formation of 15-HETE as detected by reverse-phase HPLC analysis. In contrast, markedly reduced or no 15-HETE production was detected in simultaneously incubated samples of prostate adenocarcinoma (n = 3; Figure 3).

Reduced 15-LOX-2 in Prostate Adenocarcinoma by Immunohistochemistry

In contrast to the strong, uniform 15-LOX-2 immunostaining in benign prostate glands (particularly in the peripheral zone), 15-LOX-2 immunostaining was markedly reduced in prostate adenocarcinoma (Figure 4). 15-LOX-2 immunostaining was at least focally absent in essentially all tumors, with the vast majority showing substantial portions of tumor that were negative on immunostaining. In 14 of 18 cases, 15-LOX-2 was absent in more than 25% of the tumor examined by immunohistochemistry. In these cases, 15-LOX-2 immunostaining was negative in 74.9 ± 24.4% of the tumor. 15-LOX-2 was absent in >50% of the tumor in 10 of 18 cases and essentially completely absent in 8 of 18 of the tumors.

Correlation of reduced 15-LOX-2 with other known prognostic factors in prostate cancer, such as grade and stage, and clinical outcome will require analysis of a much larger number of cases, which currently is in progress. However, possible correlation of reduced 15-LOX-2 immunostaining with increased tumor grade or biological aggressiveness was indicated in several individual cases. In one case of a Gleason 3 + 3 = 6 adenocarcinoma with absent immunostaining in greater than one-half of the tumor assessed by immunohistochemistry, there was strong residual 15-LOX-2 immunopositivity in a Gleason pattern 2 tumor focus in the transition zone (Figure 4, C and D). In two cases of overall Gleason 7 adenocarcinoma (one 3 + 4 and one 4 + 3), areas of Gleason pattern 2 and 3 were 15-LOX-2 immunopositive, whereas other areas of Gleason patterns 3 and 4 were negative, including in one case areas where the tumor was penetrating beyond the prostate capsule (Figure 4, E–G). These two cases were among those assessed by HPLC, the tumor tissue for which was taken from the posterior peripheral zone (see also Figure 5, A and B). Lack of 15-HETE formation from this tumor tissue likely correlates with the reduced 15-LOX-2 immunostaining in more peripheral (and higher-grade) portions of the tumor. Another example of possible tumor heterogeneity with regard to 15-LOX-2 expression was seen in a case with a mixed ductal and acinar pattern. Whereas the more peripherally located acinar carcinoma was largely 15-LOX-2 immunopositive, the more centrally located ductal carcinoma was negative (Figure 4, H and I). Examples of the degree of heterogeneity of loss of 15-LOX-2 immunostaining within individual tumors are shown in Figure 5, in which parts A–C correspond to the tumor cases analyzed by HPLC (as shown in Figure 3). Figure 5D shows an extreme example of the multifocal or heterogeneous nature of 15-LOX-2 negativity seen in some cases with only partial loss of 15-LOX-2 immunostaining.

Discussion

As discussed more fully below, there have been a few previous reports on the occurrence of AA-metabolizing enzymes in benign prostate and prostatic neoplasia. These include the arachidonate 5-lipoxygenase,[11] 12-lipoxygenase,[10] and reticulocyte type of 15-lipoxygenase (15-LOX-1).[12] In this study we have used assays of enzyme activity and immunohistochemistry to document expression of the recently described 15-lipoxygenase enzyme 15-LOX-2[8] in benign human prostate epithelium and its reduction in prostate adenocarcinoma. One clear advantage of the methodological approach in our study is the ability to correlate enzyme protein with actual functional activity.

Characterization of the 15-lipoxygenase metabolism and its designation as 15-LOX-2 is based on the following lines of evidence. 1) Previous multitissue Northern blots demonstrated 15-LOX-2 mRNA expression in benign prostate,[8] and we have recently detected 15-LOX-2 mRNA in individual benign prostates on Northern Blots (S. B. Shappell, unpublished observations). 2) Analysis of lipoxygenase product formation by HPLC identifies the specific formation of 15-HETE in all of our incubations of benign prostate. Typically, the reticulocyte type of 15-lipoxygenase, 15-LOX-1, forms a mixture of 15-HETE and 12-HETE,[17] whereas 15-LOX-2 oxygenates AA solely at C-15.[8] In our experiments the characteristics of 15-HETE formation matched the catalytic activities of 15-LOX-2 (eg, Figure 1 and Figure 3, A and C). The exception, when both 12- and 15-HETE formation was evident (Figure 3B), might be accounted for by co-expression of 12-LOX and a 15-lipoxygenase. 3) An additional feature of the 15-lipoxygenase metabolism in prostate, again characteristic of 15-LOX-2, was the greater formation of 15-HETE from AA than of 13-hydroxyoctadecadienoic acid (13-HODE) from linoleic acid in side-by-side incubations of the same benign prostate tissue (not shown); this correlates with the substrate specificity of 15-LOX-2[8] and contrasts with the known preference of 15-LOX-1 for linoleic over arachidonic acid.[18] 4) The antibody used in the current immunohistochemical studies specifically detects 15-LOX-2 and does not cross-react with 15-LOX-1,[19] 12S-LOX, or 5-LOX (results herein). Furthermore, there was complete suppression of the positive reactions in the immunohistochemical staining by preabsorption with purified 15-LOX-2 protein. The specificity of our antibody for 15-LOX-2 *versus* 15-LOX-1 was also supported by the staining of the prostatic epithelium with concomitant lack of staining of inflammatory cells, including macrophages, which might be expected to stain for 15-LOX-1.[20] Although inflammation was variable and generally mild in the sections examined, one benign prostate examined by both enzyme assay and immunostaining was a cystoprostatectomy specimen containing granulomatous inflammation secondary to previous BCG instillation therapy for bladder transitional cell carcinoma. In addition to formation of large amounts of 15-HETE on incubation of this prostate tissue with AA, by immunohistochemistry, the anti-15-LOX-2 antibody uniformly stained benign prostate epithelium but did not stain the

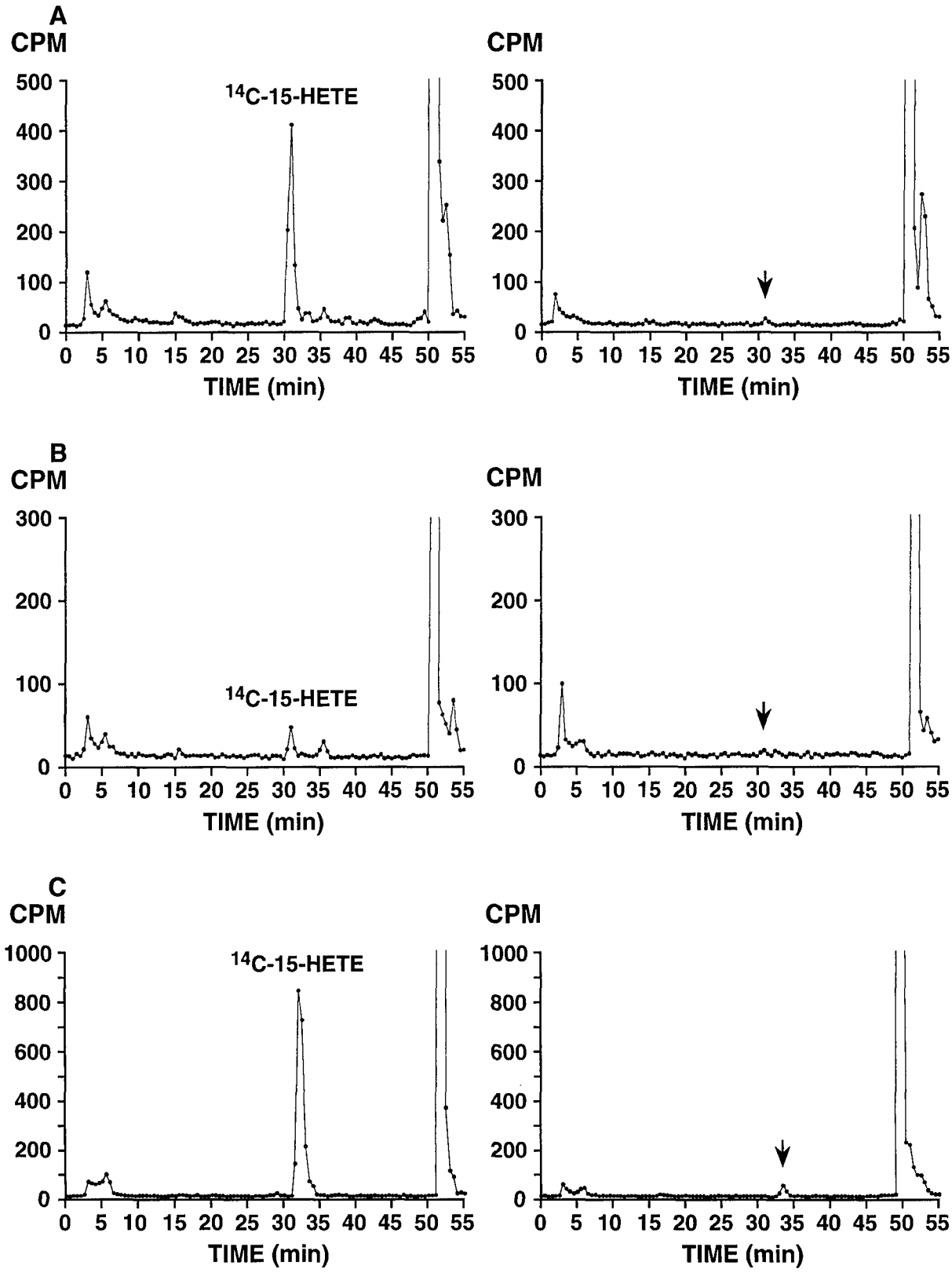

Figure 3. 15-HETE formation by benign (left) and malignant (right) prostate tissue from three different radical prostatectomy specimens. A: Benign and Gleason grade 3 + 4 = 7, with extra-capsular extension; B: Benign and Gleason grade 4 + 3 = 7, confined to prostate; C: Benign and Gleason grade 3 + 2 = 5, confined to prostate (with incubated tumor tissue from Gleason pattern 3 tumor in peripheral zone). Incubation with [^{14}C]AA and extraction were performed as described in Materials and Methods. Product analysis was by reverse-phase HPLC using a Beckman Ultrasphere 5-μm ODS column (25 × 0.46 cm) with a solvent of methanol/water/glacial acetic acid (75:25:0.01, by volume) at a flow rate of 1.01 ml/minute switched to 100% methanol at 40 minutes; 0.5-minute fractions were collected and subjected to scintillation counting. [^{14}C]15-HETE peaks are indicated in benign incubations and arrows corresponding to substantially reduced or undetected [^{14}C]15-HETE in tumor incubations based on retention time of unlabeled HETE standards co-injected with ^{14}C samples.

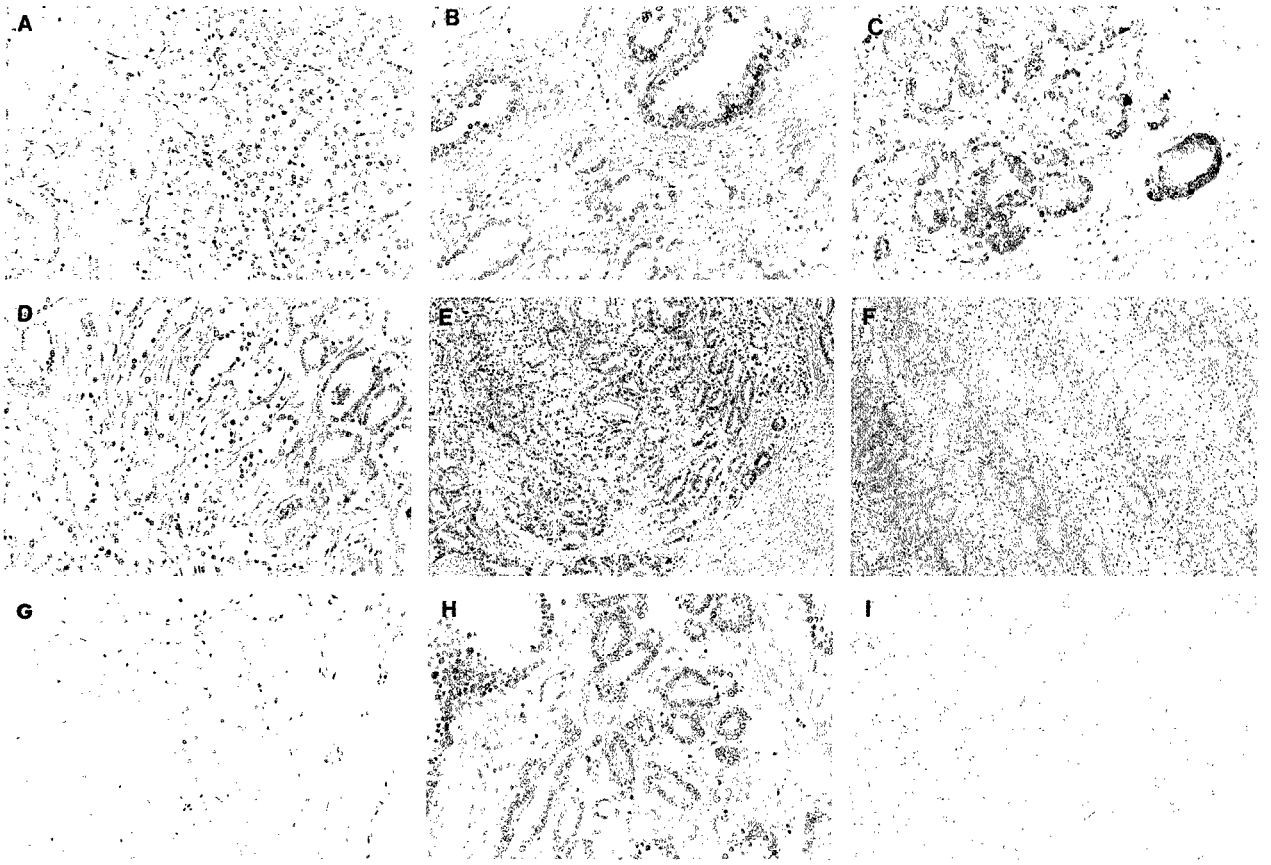

Figure 4. Reduced 15-LOX-2 immunostaining in prostate adenocarcinoma. A: Absent immunostaining in adenocarcinoma, from tumor that was overall 39% negative for 15-LOX-2 immunostaining (original magnification, ×200). B: Absent 15-LOX-2 immunostaining in adenocarcinoma, bottom, compared with strong staining in adjacent benign glands (top), from a case in which most of the tumor was higher grade than that illustrated and in which the tumor was overall 94% 15-LOX-2 negative (×200). C: Strong 15-LOX-2 immunostaining in Gleason pattern 2 tumor in transition zone (×200). D: Markedly reduced and absent 15-LOX-2 immunostaining in Gleason pattern 3 adenocarcinoma from same case as C, which was overall 58.8% 15-LOX-2 tumor negative (×200). E: Positive 15-LOX-2 immunostaining in transition zone Gleason pattern 2 adenocarcinoma (×100). F: Transition between focal weakly positive adenocarcinoma, left, and 15-LOX-2 negative tumor, right, in Gleason pattern 3 and transition to area of Gleason pattern 4, respectively, from same case as E (×100). G: 15-LOX-2-negative adenocarcinoma in area of extracapsular extension, from same case as in E and F, which was overall Gleason 3 + 4 and 44.2% 15-LOX-2 tumor negative (×200). H: Positive 15-LOX-2 immunostaining in acinar adenocarcinoma from case of mixed ductal and acinar tumor (×200). I: Negative 15-LOX-2 immunostaining in ductal adenocarcinoma, same case as in H, which was overall 45% 15-LOX-2 tumor negative (×100).

large numbers of histiocytes (macrophages) forming the granulomas (not shown).

Few studies have examined the possible significance of AA metabolism or the presence of cyclooxygenase or lipoxygenase enzymes in benign prostate and prostatic neoplasia. Chaudry et al[9] reported prostaglandin E_2 (PGE_2) synthesis to be 10-fold higher in carcinoma tissue compared with benign prostatic hyperplasia (BPH) tissue. Whether the enzymes responsible for PGE_2 synthesis were epithelial, stromal, or vascular was not elucidated. Increased formation of PGE_2 has been demonstrated previously in human cancer cell lines, including human prostate cancer cell lines.[21]

More recently, multiple reports have described the presence of lipoxygenase enzymes in prostate cancer and prostatic carcinoma cell lines. Gao et al[10] reported that 12-lipoxygenase (12-LOX) mRNA was elevated in 46 of 122 (38%) prostate tumor samples compared with normal tissue from the same patient. They found that 12-LOX mRNA was more likely to be elevated in tumor *versus* control tissue in patients with extracapsular extension (T3), positive lymph nodes, higher-grade lesions (Gleason score ≥ 8), and positive surgical margins. By *in situ* hybridization they detected 12-LOX mRNA within epithelial cells of the prostate, including basal cells of normal prostate tissue. Secretory cells were generally negative in normal tissue. This basal cell distribution of 12*S*-LOX in benign prostate tissue contrasts with the apical cell distribution of 15-LOX-2 in benign prostate demonstrated by immunohistochemistry herein. In contrast to benign glands, Gao et al reported that tumor epithelial cells demonstrated heterogeneous and occasionally marked expression of 12-LOX mRNA.[10] Biological effects of 12-HETE include promotion of tumor cell adhesion and endothelial cell contraction, indicating a potential contribution to tumor cell metastasis[2,22,23] and possible modulation of tumor growth by induction of angiogenesis.[24] 12-LOX enzyme activity has not been reported in actual prostate tissues or prostate cancers, however. We did not observe detectable 12-HETE accumulation in most incubations of benign prostate tissue with exogenous arachidonic acid. We also did not observe 12-HETE formation in similar incubations with prostate adenocarcinoma. However, although the cases ex-

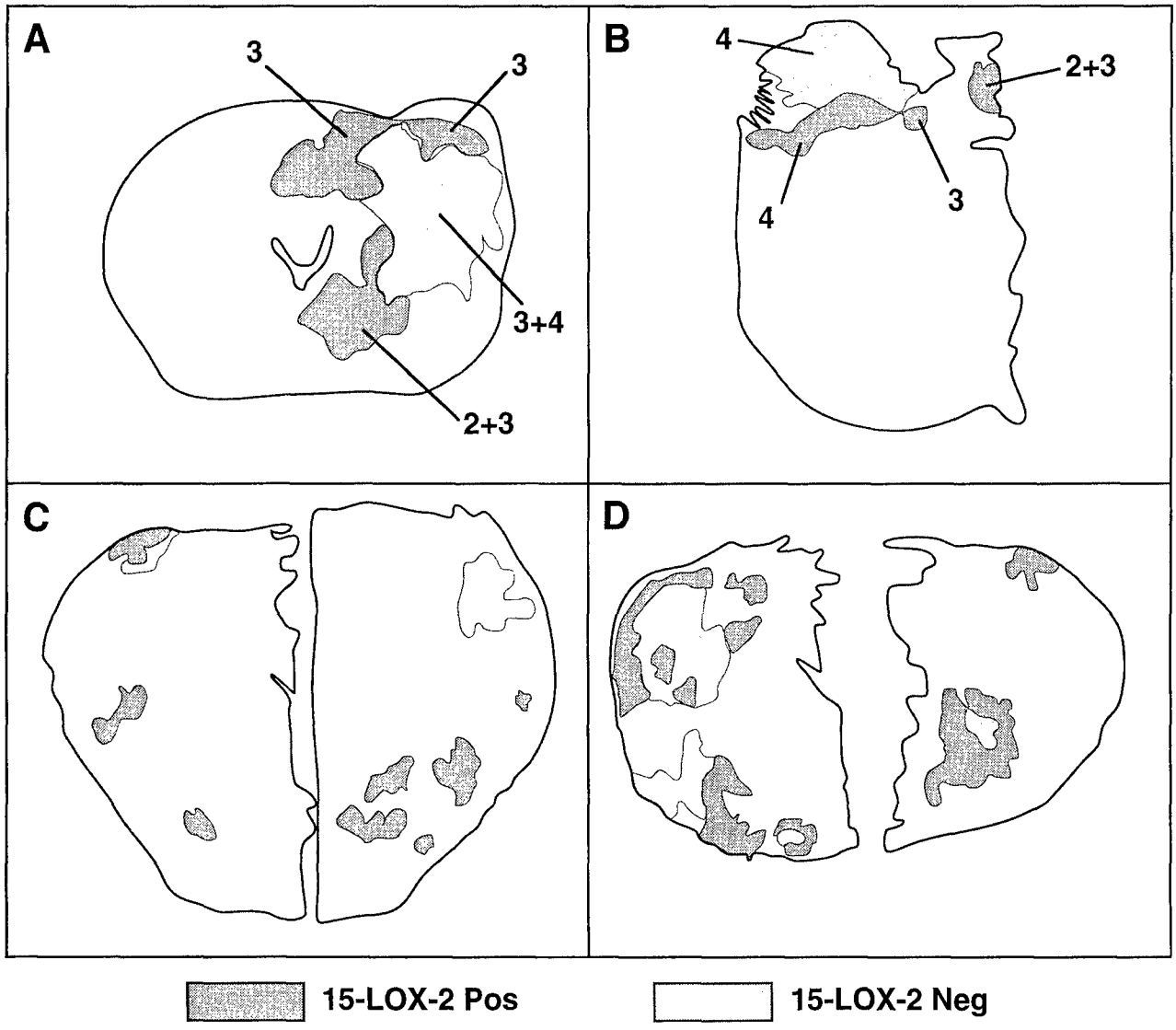

Figure 5. Heterogeneity of absent 15-LOX-2 immunostaining in prostate tumors. Immunostained entire whole-mount sections or right and/or left halves from four representative cases are outlined, with posterior toward top of figure. Benign glands stained uniformly, and stroma was negative as described in Results. Shaded outlines correspond only to actual tumor portions, with positive staining in dark gray and absent immunostaining areas in lighter shade. Numbers correspond to Gleason pattern of indicated areas (in tumors with more than one pattern). A through C correspond to cases A through C in Figure 3 (in which portions were also examined by HPLC assay). A: Gleason 3 + 4 = 7, overall 44.2% 15-LOX-2 negative. B: Gleason 4 + 3 = 7, overall 60.6% 15-LOX-2 negative. C: Gleason 3 + 3 = 6, overall 40% 15-LOX-2 negative. D: Gleason 3 + 3 = 6, overall 45% 15-LOX-2 negative.

amined for 15-LOX-2 immunostaining included multiple examples of high-grade (Gleason ≥ 8) and T3 carcinoma, only one of the cases examined by HPLC was positive for extracapsular extension (T3), and none had positive surgical margins or lymph node metastases. Hence, the possibility of detecting 12-HETE formation and possible simultaneous decreased 15-HETE and increased 12-HETE formation under the experimental conditions used will require examination of higher-grade and higher-stage lesions.

A possible role for 5-LOX in modulation of growth of the prostate cancer cell line PC-3 was reported by Ghosh and Myers.[11] Arachidonic acid stimulated PC-3 cell growth, which was inhibited by selective 5-LOX inhibitors but not by 12-LOX or cyclooxygenase inhibitors. Furthermore, 5-HETE was produced from exogenous AA in PC-3 cells.[11] More recently, these investigators and others have demonstrated induction of apoptosis in PC-3 and LNCaP cell lines by inhibitors of 5-LOX and 5-LOX-activating protein (FLAP).[25,26] Again, we did not observe 5-HETE production from exogenous AA in benign prostate or a limited number of fresh-frozen prostate tumors. Given the known chromosomal and other possible genetic abnormalities of established prostate carcinoma cell lines, whether AA metabolic pathways in these cultured cells generated from individual tumors reflect similar alterations in actual cancers *in vivo* will require correlative studies, with analysis of high-grade and high-stage cancers, perhaps including metastatic tumors.

Spindler et al[12] recently provided evidence for a possible 15-lipoxygenase in the human prostate cancer cell lines LNCaP and PC-3, which they indicated as the same

as the rabbit reticulocyte 15-lipoxygenase (designated herein as 15-LOX-1). These findings may require re-evaluation in light of the recent discovery of 15-LOX-2. However, based on the evidence we have obtained in human prostate cancer samples, transformed prostate carcinoma cell lines, such as LNCaP and PC-3 cells, would not be expected to express 15-LOX-2. The reported detection of 13-HODE in a single specimen of human prostate carcinoma by frozen-section immunohistochemistry (and its absence in surrounding benign epithelium)[12] does not correlate with the patterns of 15-LOX-2 expression observed in the series of samples we have examined, although 15-LOX-2 was still present to some degree by immunohistochemistry in a significant percentage of tumor cases. Certainly the origins of immunodetectable 13-HODE in tumor tissue might include its production through non-enzymatic mechanisms.

The biological significance of 15-LOX-2 expression in benign prostatic epithelium is not clear. The uniform expression in prostate apical or secretory cells suggests a possible role of this novel enzyme in secretory function. Reduced expression in atrophic prostate glands and prostate adenocarcinoma may thus parallel less secretory differentiation. However, given our demonstration of the loss of this protein and its associated enzymatic activity in prostate carcinoma, it is possible that reduction or loss of 15-LOX-2 and 15-HETE formation is a crucial event in the development or progression of prostate adenocarcinoma. In this regard, it is noteworthy that the murine homologue of 15-LOX-2, a recently identified 8*S*-lipoxygenase (8-LOX), is up-regulated in benign squamous papilloma of mouse skin induced by phorbol ester treatment and lost with progression to frank squamous cell carcinoma with additional treatments.[19,27] It is possible that the products of these related enzymes (15*S*-HETE and 8*S*-HETE, respectively) regulate aspects of cellular proliferation or differentiation and that the loss of this function is associated with development or progression of a malignant phenotype. The recent description of the activation of transcription-regulating PPAR receptor subtypes (peroxisome proliferator-activated receptors) by various AA-related products, including HETEs, indicates one potential mechanism of action.[28–31] This is a particularly intriguing hypothesis in light of the evidence that the synthetic activators of PPAR, the aromatic fatty acids phenyl acetate and phenyl butyrate, inhibit prostate cancer cell line growth *in vitro* and show activity against hormone-insensitive prostate cancer *in vivo*.[32–34] More recently, Kubota et al[35] demonstrated expression of PPARγ in PC-3, DU-145, and LNCaP cells and inhibition of PC-3 cell proliferation by PPARγ ligands, including the thiazolidinedione troglitazone and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2. The recent demonstration of activation of PPARγ-dependent transcription by a variety of oxidized lipids, including specifically 15-HETE,[36] suggests a possible role of reduced 15-HETE activation of PPARγ in prostate carcinogenesis or progression. Whether this signaling pathway or some other mechanism of cell regulation is affected by alterations of prostate 15-LOX-2 expression remains to be further clarified.

Acknowledgments

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